PARASITOLOGY

PARASITOLOGY

(Founded by G. H. F. Nuttall, F.R.S.)

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CONTENTS

Nos. 1, 2 (May 1960)

PAGE

JAIN, G. P. On the genus *Artyfechinostomum* (Lane, 1915) (Echinostomatidae: Trematoda) 1

JAIN, G. P. Further observations on *Artyfechinostomum mehrai* (Faruqui) (Echinostomatidae: Trematoda). (With 6 Figures in the Text) 7

KULASIRI, C. The indicator system of the Sabin-Feldman phenomenon in *Toxoplasma* 13

EUZET, L. and HARFORD WILLIAMS, H. A re-description of the trematode *Calicotyle stossichii* Braun, 1899, with an account of *Calicotyle palombi* sp.nov. (With 11 Figures in the Text) 21

THOMAS, R. J. and STEVENS, A. J. Ecological studies on the development of the pasture stages of *Nematodirus battus* and *N. filicollis*, nematode parasites of sheep. (With 5 Figures in the Text) 31

LLEWELLYN, J. and OWEN, I. L. The attachment of the monogenean *Discocotyle sagittata* Leuckart to the gills of *Salmo trutta* L. (With 1 Plate and 4 Figures in the Text) 51

REES, B. *Albertia vermicularis* (Rotifera) parasitic in the earthworm *Allolobophora caliginosa*. (With 1 Plate and 4 Figures in the Text) 61

SEAMER, J. Studies with *Aperythrozoon parvum* Splitter, 1950. (With 1 Figure in the Text) 67

WALTON, G. A. The reaction of some variants of *Ornithodoros moubata* Murray (Argasidae, Ixodoidea) to desiccation. (With 2 Figures in the Text) . . 81

JACK, K. M. *Geckobia japonicum*, a re-publication of *Pterygosoma japonicum* Kishida, 1927. (Acarina, Pterygosomidae). (With 1 Figure in the Text) . 89

OSBORNE, P. Observations on the natural enemies of *Meligethes aeneus* (F.) and *M. viridescens* (F.) [Coleoptera: Nitidulidae]. (With 16 Figures in the Text) 91

CORLISS, J. O. *Tetrahymena chironomi* sp.nov., a ciliate from midge larvae, and the current status of facultative parasitism in the genus *Tetrahymena*. (With 7 Figures in the Text) 111

HANUMANTHA RAO, K. Studies on *Penetrocephalus ganapatii*, a new genus (Cestoda: Pseudophyllidea) from the marine teleost *Saurida tumbil* (Bloch). (With 1 Plate and 8 Figures in the Text) 155

PANTELOURIS, E. M. and GRESSON, R. A. R. Autoradiographic studies on *Fasciola hepatica* L. (With 1 Plate) 165

MCCONNACHIE, ELSPETH W. Experiments on the encystation of *Opalina* in *Rana temporaria* 171

SPRENT, J. F. A. and MINES, J. J. A new species of *Amplicaecum* (Nematoda) from the carpet snake (*Morelia argus variegatus*): with a re-definition and a key for the genus. (With 20 Figures in the Text) 183

PAGE

ARTHUR, D. R. A review of some ticks (Acarina: Ixodidae) of sea birds. Part II. The taxonomic problems associated with the *Ixodes auritulus-percavatus* group of species. (With 78 Figures in the Text) 199

ARTHUR, D. R. A review of some ticks (Acarina: Ixodidae) of sea birds. Part III. A re-description of the male of *Ixodes kerguelenensis* André & Colas-Belcour, 1942. (With 8 Figures in the Text) 227

INGRAM, D. G. and SOLTYS, M. A. Immunity in trypanosomiasis. Part IV. Immunoconglutinin in animals infected with *Trypanosoma brucei* . . 231

LEE, D. L. The effect of changes in the osmotic pressure upon *Hammerschmidtiella diesingi* (Hammerschmidt, 1838) with reference to the survival of the nematode during moulting of the cockroach. (With 4 Figures in the Text) . . 241

LEE, D. L. The distribution of glycogen and fat in *Thelastoma bulhõesi* (Magalhães, 1900), a nematode parasitic in cockroaches. (With 24 Figures in the Text) . 247

SOMMERVILLE, R. I. The growth of *Cooperia curticei* (Giles, 1892), a nematode parasite of sheep. (With 2 Figures in the Text) 261

JOHRI, G. N. A new paruterinid cestode, *Lallum magniparuterina* gen. et sp.nov. from the intestine of a common teal, *Nettion crecca* Linn. 269

HARRINGTON, J. S. A simple apparatus for the artificial feeding of *Rhodnius prolixus* (Hemiptera, Reduviidae) 273

HARRINGTON, J. S. Studies on *Rhodnius prolixus*: growth and development of normal and sterile bugs, and the symbiotic relationship 279

NOS. 3, 4 (NOVEMBER 1960)

BAUGH, SANJIB CHANDRA. Studies on Indian Rhynchobdellid leeches. I. (With 6 Figures in the Text) 287

HARFORD WILLIAMS, H. Some observations on *Parabothrium gadi-pollachii* (Rudolphi, 1810) and *Abothrium gadi* van Beneden 1870 (Cestoda: Pseudophyllidea) including an account of their mode of attachment and of variation in the two species. (With 40 Figures in the Text) 303

PANDE, B. P., AHLUWALIA, S. S. and SRIVASTAVA, J. S. Note on host-parasite relationships observed in fluke infections of wild aquatic birds. (With 5 Figures in the Text) 323

ROGERS, W. P. and SOMMERVILLE, R. I. The physiology of the second ecdysis of parasitic nematodes. (With 1 Plate and 11 Figures in the Text) . . . 329

HANUMANTHA-RAO, K. The problem of Mehlis's gland in helminths with special reference to *Penetrocephalus ganapatii* (Cestoda: Pseudophyllidea) . . 349

VICKERMAN, K. *Herpetomonas ludwigi* (Kramář, 1950) n.comb., the trypanosomatid parasite of cranefly larvae (Diptera, Tipulidae). (With 5 Figures in the Text) 351

THEODOR, O. and COSTA, M. New species and new records of Argasidae from Israel. Observations on the rudimentary scutum and the respiratory system of the larvae of the Argasidae. (With 28 Figures in the Text) 365

PAGE

FIELD, A. C., BRAMBELL, M. R. and CAMPBELL, J. ALLAN. Spring rise in faecal worm-egg counts of housed sheep, and its importance in nutritional experiments. (With 3 Figures in the Text) 387

ILES, CHRISTINE. The larval trematodes of certain fresh-water molluscs. (With 1 plate) 401

KULASIRI, C. The nature of the cytoplasm-modifying antibodies. (With 2 Figures in the Text) 419

MAWSON, PATRICIA M. Two new species of trichostrongyle nematodes from the Australian pouched mouse, *Phascogale flavipes* (Waterhouse). (With 11 Figures in the Text) 425

BISHOP, ANN and MCCONNACHIE, ELSPETH W. Further observations on the *in vitro* development of the gametocytes of *Plasmodium gallinaceum* . . 431

VINCENT, PATRICIA and NEAL, R. A. Duration of invasiveness of *Entamoeba histolytica* maintained *in vitro*. (With 1 Figure in the Text) 449

RAWSON, DILYS and RIGBY, JOYCE E. The functional anatomy of the cysticercoid of *Choanotaenia crassiscolex* (Linstow 1890) (Dilepididae) from the digestive gland of *Oxychilus cellarius* (Müll) (Stylommatophora), with some observations on developmental stages. (With 1 Plate and 5 Figures in the Text) . 453

DINNIK, J. A. and DINNIK, N. N. Development of *Carmyerius exoporus* Maplestone (Trematoda: Gastrothylacidae) in a snail host. (With 8 Figures in the Text) 469

TATCHELL, R. J. A comparative account of the tracheal system of larvae of the horse bot-fly, *Gasterophilus intestinalis* (De Geer), and of some other dipterous larvae. (With 16 Figures in the Text) 481

PRINGLE, G. Invasion of the egg masses of the mollusc *Bulinus* (*Physopsis*) *globosus*, by larval chironomids 497

DASGUPTA, B. Lipids in different stages of the life cycles of malaria parasites and some other sporozoa. (With 2 Figures in the Text) 501

DASGUPTA, B. Polysaccharides in the different stages of the life-cycles of certain sporozoa. (With 3 Figures in the Text) 509

BAKER, J. R. Trypanosomes and dactylosomes from the blood of fresh-water fish in East Africa. (With 1 Plate and 2 Figures in the Text) 515

TATE, P. Notes on *Limnophora exsurda* Pand. (Diptera, Anthomyidae). (With 7 Figures in the Text) 527

NEAL, R. A. Enzymic proteolysis by *Entamoeba histolytica*: biochemical characteristics and relationship with invasiveness. (With 10 Figures in the Text) . 531

NASIR, P. Trematode parasites of snails from Edgbaston Pool: the life history of the strigeid *Cotylurus brevis* Dubois & Rausch, 1950. (With 8 Figures in the Text) 551

AH, HYONG-SUN. *Shunsennia gracilis*, a new species of Trombiculid Mite from Korea (Acarina, Trombiculidae). (With 1 Figure in the Text) 577

GANAPATI, P. N. and NARASIMHAMURTI, C. C. On a new haplosporidian (protozoa) *Nephridiophaga xenoboli* in the gut of a millipede *Xenobolus carnifex*. (With 9 Figures in the Text) 581

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On the genus *Artyfechinostomum* (Lane, 1915) (Echinostomatidae: Trematoda)

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(*Received* 3 *October* 1958)

INTRODUCTION

Artyfechinostomum Lane, 1915 (type *A. sufrartyfex* Lane, 1915) is a synonym of *Paryphostomum* Dietz, 1909 (type *P. radiatum* Dujardin, 1845). While the general morphology of the two forms is similar and places them both in the family Echinostomatidae, other characters clearly place them in different genera of this family. In *Artyfechinostomum* the cirrus-sac extends far posterior to the acetabulum, and the right and left fields of vitellaria in the post-testicular region are confluent. In *Paryphostomum* the cirrus-sac is small and anterior to the middle of the acetabulum and the vitellaria in the post-testicular region are not confluent. These differences entitle *Artyfechinostomum* to be placed in a separate genus. The species *Paryphostomum indicum* Bhalerao, 1931 and *P. mehrai* Jain, 1957 are transferred to the genus *Artyfechinostomum*.

KEY TO GENERA

1. Collar spines 27–39 in number. Cirrus-sac small, dorsal to acetabulum.	**Paryphostomum**
2. Collar spines 39–43 in number. Cirrus-sac long, inclined over acetabulum.	**Artyfechinostomum**

Genus **Paryphostomum** Dietz, 1909

Diagnosis. Body small, somewhat narrow anteriorly and posteriorly. Collar beset with 27–39 collar spines arranged in single or double rows. Cuticular spines arranged in transverse rows, not extending beyond ventral sucker posteriorly. Ventral sucker globular or elongated posteriorly and much larger than oral sucker. Testes with 3–5 (mostly 4–5) lobes, one behind the other. Cirrus-sac moderately developed, dorsal to ventral sucker. Vitellaria extending from posterior region of ventral sucker to hinder end of body and not meeting in median line behind testes. Uterus short, composed of a few coils. Eggs few or numerous.

Type-species: *Paryphostomum radiatum* (Dujardin, 1845), Dietz, 1909

Key to the species of the genus Paryphostomum *Dietz*, 1909

Collar spines 27–39 in single row.	**A**
Collar spines 27–35 in double rows.	**B**
A. Collar spines 27 in number.	**1**
Collar spines 39 in number.	**2**
1. Testes trilobed.	**P. testitrifolium** (Gogate, 1934)
Testes 4–5 lobed.	**P. radiatum** (Dujardin, 1845)
2. Testes trilobed.	**P. horai** (Baugh, 1950)

B. Collar spines 27 in number.	3
Collar spines 35 in number	4
3. Testes 4–5 lobed.	**P. segregatum** (Dietz, 1910)
4. Testes 5 lobed.	**P. pentalobum**(Verma, 1936)

Genus **Artyfechinostomum** (Lane, 1915)

Diagnosis. Body long, narrow or pointed anteriorly, flattened and rounded posteriorly. Collar armed with 39–43 collar spines arranged in single or double dorsally uninterrupted rows. Cuticular spines almost all over body or becoming sparser posteriorly to disappear completely near posterior extremity. Ventral sucker oval, sometimes funnel-shaped, and larger than oral sucker. Testes deeply lobed (mostly with 6–7 lobes). Cirrus-sac well developed, extending beyond posterior margin of ventral sucker. Ovary variable in shape, usually pear-shaped, to right side of median line anterior to testes. Vitellaria extending from middle or posterior region of ventral sucker to hinder end of body and well developed in post-testicular region. Uterus long, composed of a number of coils. Eggs few or numerous.

Type-species: *Artyfechinostomum sufrartyfex* (Lane, 1915)

Key to the species of the genus Artyfechinostomum (Lane, 1915)

1. Collar spines in single row.	**A. sufrartyfex** (Lane, 1915)
— Collar spines in double rows.	2
2. Vitellaria extend from middle of ventral sucker. Eggs few.	**A. indicum** (Bhalerao, 1931)
— Vitellaria extend from posterior region of ventral sucker. Eggs numerous.	**A. mehrai** (Faruqui), Jain, 1957.

Artyfechinostomum sufrartyfex Lane, 1915

Syn. *Euparyphium malayanum* Leiper, 1911.
Paryphostomum sufrartyfex (Lane, 1915) Bhalerao, 1931.

Hosts. Man, pig. *Location.* Intestine. *Locality.* Assam, Bengal.

Diagnosis. Shape of body variable, 9·0 mm. long, 2·5 mm. broad. Collar armed with 39 collar spines disposed in single, dorsally-uninterrupted row. Whole of ventral surface covered with cuticular spines. Ventral sucker larger than oral sucker, ratio of suckers 1:5. Pre-pharynx short, pharynx 0·18–0·35 mm. long, oesophagus 0·35 mm. long. Testes deeply lobed. Cirrus-sac, 2 mm. long, extending 0·75 mm. behind ventral sucker. Cirrus present without spines. Ovary oval, to right side of body, anterior to testes. Vitellaria extending from posterior border of ventral sucker to hinder end of body meeting dorsally in median line behind testes. Uterus long, with compact coils. Eggs few; 0·058–0·075 × 0·09–0·113 mm.

Artyfechinostomum indicum (Bhalerao)

Syn. *Testisaculus indicum* Bhalerao, 1927
Paryphostomum indicum Bhalerao, 1931

Host. Uromastix hardwickii. Location. Duodenum. *Locality.* North India.

Diagnosis. Body elongated, somewhat eliptical, 7·5–8·1 mm. long. Collar beset with 42 spines arranged in double, dorsally-uninterrupted rows. Cuticular spines spreading from the anterior end to region of ovary. Ventral sucker larger than oral sucker, their ratio 1:3·5. Testes 3–7 lobed; cirrus-sac elongated, extending up

to posterior margin of ventral sucker. Ovary oval, on right side anterior to testes. Vitellaria, extending from middle of ventral sucker to hinder end of body, meeting each other in median line in post-testicular region. Uterus with few coils. Eggs few, 0·041–0·097 × 0·033–0·072 mm.

Artyfechinostomum mehrai (Faruqui)

Syn. *Paryphostomum mehrai* (Faruqui; Jain, 1957).

Host. White rat. *Location.* Intestine. *Locality.* Allahabad.

Diagnosis. Body narrow anteriorly, broad and rounded posteriorly, broadest in the testicular region, 8·36 mm. long. Musculature well developed. Collar beset with 43 spines arranged in double rows not interrupted dorsally. Cuticle spiny, spines arranged in transverse rows all over body, except hindermost region.

Table 1. *Comparative table of the species belonging to the genus* Paryphostomum (*Dietz*)

Species ...	*P. radiatum* (Dujardin)	*P. segregatum* (Dietz)	*P. testitrifolium* (Gogate)	*P. horai* (Baugh)	*P. pentalobum* (Verma)
Host ...	*Plecanus carbo*	*Cathartes urubutinga*	*Dendrocygma javanica*	*Anas poecilorhyncha*	*Snipe*
Size (mm.)	3·3–6·5	5·75–8·8	3·5–5·0	3·05–0·61	18·5
Collar spines	27 in single row	27 in double rows	27 in single row	39 in single row	35 in double rows
Cuticular spines	Pre-acetabular	Pre-acetabular	Pre-acetabular	Pre-acetabular	Spiny cuticle
Ratio of suckers	1:3	1:3	1:5	1:3·8	1:3
Testes	4–5 lobed	4–5 lobed	3 lobed	3 lobed	5 lobed
Cirrus-sac	Small, dorsal to ventral sucker	Small	Small	Small	Small
Vitelline follicles	Not meeting in median line behind testes	A similar condition is found in all other forms			

Table 2. *Comparative table of the species belonging to the genus* Artyfechinostomum (*Lane*)

Species ...	*A. sufrartyfex* (Lane)	*A. indicum* (Bhalerao)	*A. mehrai* (Faruqui)
Host ...	Pigs	*Uromastix hardwicki*	White rat
Size (mm.)	9·0	7·5–8·1	8·36
Collar spines	39 in single row	42 in double rows	43 in double rows
Cuticular spines	Cuticle spiny	Spiny	Spiny
Ratio of suckers	1:5	1:3·5	1:3·5
Testes	6–7 lobed	3–7 lobed	5–6 lobed
Cirrus-sac	Elongated, extending up to posterior margin of ventral sucker in all these forms		
Vitelline follicles	Meeting in the median line in all forms		

Ratio of two suckers 1:3·5. Prepharynx short, 0·069 mm. long; pharynx 0·24 mm., oesophagus 0·22 mm. long. Testes deeply lobed, mostly divided into 5–6 lobes. Cirrus-sac 1·98 mm. long, extending posteriorly beyond acetabulum. Vesicula seminalis 0·994 mm. long, filling greater portion of cirrus sac, opening through a minute aperture by a fairly wide long duct into pars prostatica. Ovary pear-shaped, 0·44 mm. in diameter. Vitellaria extending from posterior region of acetabulum to hinder end of body, well developed in median line behind testes. Uterus with few coils. Eggs numerous, 0·096 × 0·064 mm.

Remarks. Lane in 1915 described a new form, *Artyfechinostomum sufrartyfex*, from the intestine of a girl in Assam, creating for this species the new genus *Artyfechinostomum*. The validity of this genus was, however, questioned and the species described by Lane, *A. sufrartyfex* (Lane), was included by some workers in the genus *Echinostoma* Rudolphi, 1809 and by others in the genus *Euparyphium* Dietz, 1909. In 1915, Leiper and Odhner were led to think that *Artyfechinostomum sufrartyfex* Lane, 1915 is a synonym of *Euparyphium malayanum* (Leiper, 1911). Two years later, i.e. 1917, Lane pointed out clearly that *A. sufrartyfex* was different from *E. malayanum*. Fuhrmann (1928) considered that the genus *Artyfechinostomum* was valid, because the cirrus-sac extends some distance behind the ventral sucker, whereas in *Euparyphium* it does not extend behind this sucker and he did not, therefore, consider that these two genera are identical. Faust (1929) re-examined the co-type material of Lane's species deposited in the Indian Museum, Calcutta, and reported that *Artyfechinostomum sufrartyfex* stood apart from *Euparyphium*. Bhalerao (1931) redescribed this species from the material obtained from pigs at Calcutta and considered it to be a synonym of *Paryphostomum* Dietz, 1909.

Fuhrmann (1928) listed the genus *Artyfechinostomum* in the subfamily *Echinostomatinae* Stiles & Hassall, 1926. Bhalerao (1931) pointed out that this genus does not belong to the subfamily *Echinostomatinae* because the collar is not united ventrally by a bridge, the cirrus-sac extends beyond the ventral sucker and the vesicula seminalis is not coiled. Lane (1915) had, however, assigned it to the subfamily *Himasthlinae* Odhner, 1910. Bhalerao (1931), who regarded it as *Paryphostomum* (Dietz), also ascribed it to this subfamily. Mendheim (1943) revised the family *Echinostomatidae* Poche, 1926 and he correctly created a new subfamily *Paryphostominae* for the genus *Paryphostomum* (Dietz). It appears from Mendheim's (1943) description of the genus *Paryphostomum* Dietz, 1909 that Bhalerao (1931) was not justified in including the species *Artyfechinostomum sufrartyfex* Lane, 1915 and *Testisaculus indicum* Bhalerao, 1927 under the genus *Paryphostomum*. I am of the opinion that the genus *Artyfechinostomum* (Lane) should not be treated as a synonym of the genus *Paryphostomum* (Dietz), on account of the large number of collar spines (39–43), the long cirrus-sac inclined over the ventral sucker and the vitelline follicles meeting in the median line behind the testes. The two genera resemble each other in having the testes deeply lobed and the vitelline follicles commencing from the posterior margin of the ventral sucker. *Artyfechinostomum* (Lane) is, therefore, assigned to the subfamily *Paryphostominae* Mendheim, 1943. In view of the above, there can be no doubt about the validity of the genus *Artyfechinostomum* (Lane, 1915).

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Further observations on *Artyfechinostomum mehrai* (Faruqui) (Echinostomatidae: Trematoda)

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(*Received* 3 *October* 1958)

INTRODUCTION

While working on the life cycle of *Cercaria mehrai* Faruqui, 1930, the author obtained the adults from the intestine of experimentally infected white rats. The trematodes thus obtained were reported as belonging to the genus *Paryphostomum* Dietz, 1909. In view of the facts that the collar spines are 43 in number, the vitellaria are confluent in the post-testicular region and the cirrus-sac extends far behind the acetabulum, *P. mehrai* is transferred to the genus *Artyfechinostomum* Lane, 1915, which is, in author's opinion, a valid genus (Jain, 1959).

This paper records further observations on *A. mehrai*. About 50 stained specimens were studied and these showed interesting morphological variations.

Artyfechinostomum mehrai (Faruqui)

The trematodes (Fig. 1) are elongated, flat and almost rounded posteriorly. The body size varies considerably, ranging from 4·84 to 8·36 mm. in length. The arched collar at the anterior extremity is strongly developed. There are 43 collar spines, arranged in two rows. The spines in the terminal group are a little larger than the marginal ones. The end spines of a specimen of 4·84 mm. length measure $0{\cdot}039 \times 0{\cdot}006$ mm. The other terminal spines are $0{\cdot}036 \times 0{\cdot}006$ mm., while the marginal spines are $0{\cdot}027 \times 0{\cdot}006$ mm.

The integument

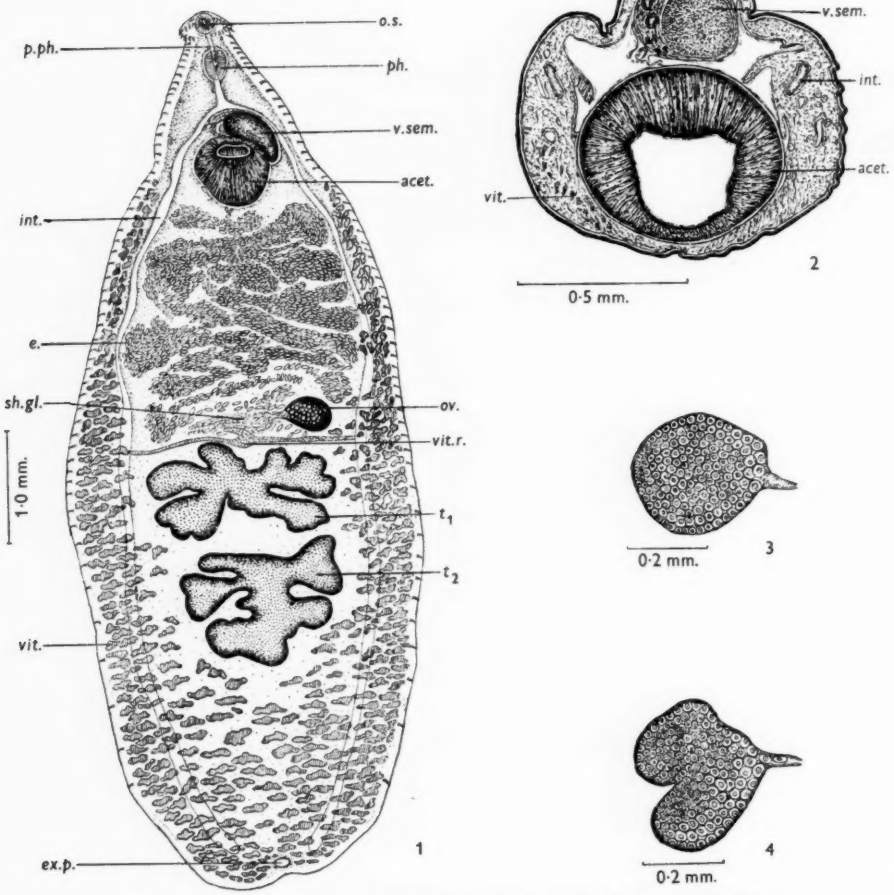
A fairly uniform cuticle covers the entire body. The integument, both ventrally and laterally, is thickly covered by a mass of small cuticular spines arranged in transverse rows directed backwards. The spines are deeply embedded in the cuticle, are of uniform size, are thick in the pre-acetabular region, and measure $0{\cdot}027 \times 0{\cdot}004$ mm. in size.

Musculature

The body has developed the usual type of trematode musculature. A single layer of circular muscle fibres lie immediately beneath the basement membrane. Beneath the circular muscles lie the thick layer of longitudinal muscle fibres beneath which are diagonal muscle fibres. The large, cutaneous gland cells occur below the diagonal muscular layer and are roughly pear-shaped, the narrow end being directed towards the outer surface; but no efferent conducting portions could be made out in sections.

Suckers

The acetabulum (Fig. 2) is well developed and muscular, with a roughly oval outline. Its position in relation to the length of body varies with the size of the worm. The cavity of the acetabulum is lined with a layer of cuticle continuous with that of the body wall. Under the cuticle is a thin fibrous membrane which separates the musculature of the sucker from it. This is followed by a thick strand of radial



Figs. 1–4. All drawings were made with the help of a camera lucida.

Fig. 1. Dorsal view of *A. mehrai* (paratype). Fig. 2. Tranverse section through ventral sucker. Fig. 3. Ovary with an entire margin. Fig. 4. Ovary showing its bilobed nature.

Abbreviations: *acet.*, acetabulum; *cir.p.*, cirrus pouch; *d.ej.*, ejaculatory duct; *ex.p.*, excretory pore; *e.*, egg; *f.g.p.*, female genital pore; *int.*, intestinal caeca; *met.*, metraterm; *m.g.p.*, male genital pore; *ov.*, ovary; *oes.*, oesophagus; *o.s.*, oral sucker; *ph.*, pharynx; *p.ph.*, prepharyx; *pr.gl.*, prostate glands; *sh.gl.*, shell gland; t_1, t_2, testes; *vit.*, vitellaria; *vit.r.*, vitelline reservoir; *v.d.*, vasa deferentia; *v.sem.*, vesicula seminalis.

muscle fibres. Underneath the radial muscle fibres lies the internal membrane closely adjacent to the parenchyma. The extrinsic musculature of the acetabulum is very well developed. Bundles of muscular and connective tissue fibres take origin from its periphery and run out radially to be attached to the dorsal, ventral and lateral regions of the body wall. The histological structure of the oral sucker is essentially the same as that of the ventral sucker, but the muscle fibres are much thinner.

Alimentary canal

The mouth is followed by a distinct prepharynx (Fig. 1), which varies in length from 0·069 to 0·224 mm. The cuticle lining the prepharynx is continuous with that of the oral sucker. The pharynx is oval in shape and appears to be histologically similar to the sucker, but its radial fibres are slightly thicker and are more closely packed. The oesophagus is lined internally by a single layer of epithelial cells, which give rise to villus-like projections. The intestinal caeca are lined throughout by a well-marked columnar type of epithelium. The caeca are attached to the body wall by fibrous bundles.

Genital system

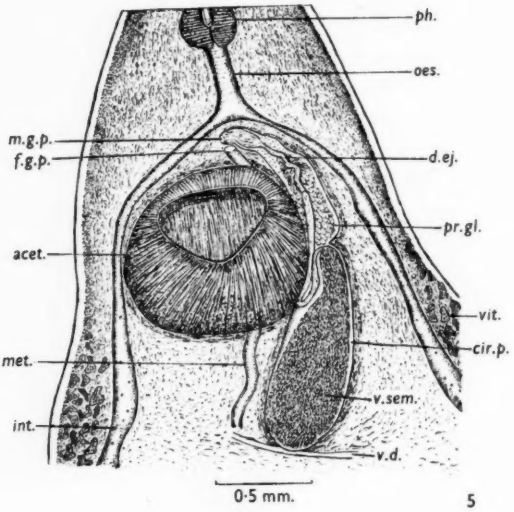
The male and female genital ducts (Fig. 5) open separately and lie closely in front of the acetabulum behind the intestinal bifurcation. The testes (Fig. 1) are lobed, and lie directly behind one another. The antero-posterior axis of the testes is 0·442–0·57 mm. and the transverse axis 0·48–0·368 mm.

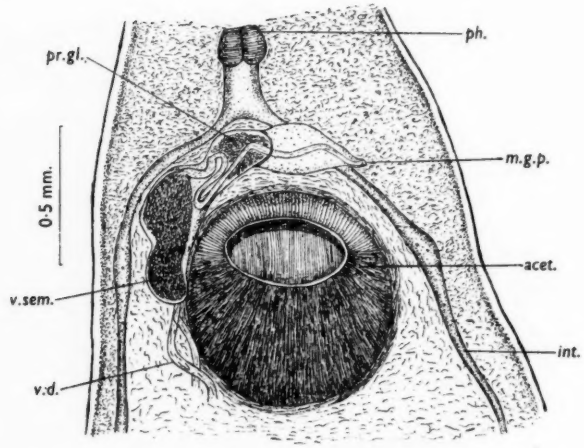
Both vasa deferentia arise from the dorsal surface of their respective testes near to the anterior end of the middle line, and run forward as far as the cirrus-sac (Fig. 6). Immediately behind the cirrus-sac they join to form the vesicula seminalis.

The cirrus-sac is elongated, with its narrow end directed forwards with a slight inclination ventrally towards the acetabulum. In some forms (Fig. 6) the cirrus-sac is found reaching up to the middle of the ventral sucker and is 0·732 mm. long, while in others it extends beyond the posterior margin of ventral sucker (Fig. 5). The vesicula seminalis is elongated and fills the greater portion of the cirrus-sac and opens by a short, 0·128 mm., duct into a long pars-prostatica (Fig. 6), which runs straight until it opens into the ejaculatory duct. In some cases the vesicula seminalis leads into a wide, elongated duct, 0·864 mm. long, which runs backwards within the pouch (Fig. 5) along the left margin of the vesicula seminalis almost up to the level of the posterior margin of the acetabulum and then runs forwards alongside the right margin of the cirrus-sac, the whole duct thus forming a V-shaped tube (Fig. 5). The pear-shaped prostatic cells form a mass surrounding the pars prostatica, into which they open by their narrow ductules. It is interesting to observe that the narrow terminal end of the cirrus-sac is in a few forms (Fig. 6) abnormal in shape.

The ovary is situated a little to the right side and is pretesticular. Its appearance varies, being in some individuals nearly pear-shaped (Fig. 3) with an entire margin, while in others it has become typically bilobed (Fig. 4). It consists of a single layer of egg cells enclosed within a basement membrane. The egg cells at the outer,

peripheral region are smaller and more compact than those in the centre and posterior part of the organ. The mature ova are somewhat oval in shape and contain a large, rounded nucleus. The oviduct arising from the inner, narrow end of the ovary is 0·16 mm. long. It passes backwards to the left side and bulges into a small, sac-like structure just before its junction with the receptaculum seminis on one side. The small receptaculum seminis is pear-shaped and is filled with sperms. The oviduct passes to the left of the shell gland, where it becomes broader and receives the narrow common yolk duct of the vitelline reservoir.





Figs. 5, 6. All drawings were made with the help of a camera lucida. For abbreviations see legend to Figs. 1–4.

Fig. 5. Cirrus-sac elongated. **Fig. 6.** Cirrus-sac short.

The compact shell gland is pear-shaped and is composed of a large number of cells which are closely aggregated together. The cells are quite discrete and are not enclosed within a membrane. They are large and are radially arranged around the ootype, into which they open by their narrow ducts.

The ootype is an elongated tube consisting of proximal narrow, and distal broadly dilated, parts; it is situated in the transverse axis and is lined internally by a layer of epithelial cells with nuclei. The vitellaria (Fig. 1) occupy the lateral margins of the body and extend from the posterior border of the ventral sucker to the hinder end of the body, overlap the intestinal caeca in the testicular region and practically unite with one another immediately behind the testes. The vitelline follicles are pear-shaped. The vitelline ducts have the usual disposition. The main ventral longitudinal ducts run one on each side of the body, parallel to the intestinal caeca. The numerous ductules from the follicles open directly into the main ducts. The transverse vitelline ducts pass inwards along the anterior border of the anterior testis to unite with one another in a small yolk reservoir which lies posterior to the shell gland.

The uterus arises from the right side of the ootype and has somewhat muscular walls. It soon passes ventrally, and then forwards, as a wide, thin-walled tube, which soon becomes coiled in a somewhat S-shaped manner, and reaches the level of the posterior end of the ventral sucker, where it opens into the metraterm. The metraterm (Fig. 5) proceeds forwards to open to the exterior at the female genital aperture, which is situated slightly posterior to the male aperture.

The ova are numerous, thin-shelled and bright yellow; they measure 0·096 × 0·064 mm.

CONCLUSION

The study of 50 specimens of adult *Artyfechinostomum mehrai* (Faruqui) obtained from the intestine of the experimentally infected white rats, revealed marked individual variation in certain characters such as the elongation of the prepharynx and of the cirrus-sac, and the lobulation of the ovary.

The writer wishes to express his appreciation of the suggestions and advice given by Professor Horace W. Stunkard and by Dr H. R. Mehra, formerly Professor of Zoology, University of Allahabad.

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The indicator system of the Sabin–Feldman phenomenon in *Toxoplasma*

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INTRODUCTION

Sabin & Feldman (1948) found that the addition of alkaline methylene blue to mouse peritoneal fluid containing *Toxoplasma*, which had been incubated with an immune serum and normal human serum, did not stain the extracellular *Toxoplasma*, although it stained the intracellular ones. When such peritoneal fluid was incubated under identical conditions with a non-immune serum, the extracellular as well as the intracellular *Toxoplasma* stained immediately on the addition of the alkaline dye. This phenomenon was the basis of the Sabin–Feldman dye test and the alkaline methylene blue constituted the indicator system of the Sabin–Feldman phenomenon. By trial and error they found that a freshly prepared solution of alkaline methylene blue was the best for optimum results. However, Cathie & Dudgeon (1949) observed that a fresh dye solution stained the *Toxoplasma* so lightly that an accurate evaluation of the stained and unstained *Toxoplasma* was not easy. They found that an alkaline dye solution which had been matured over 2 days was more suitable and that this solution was serviceable up to 14 days. Grönroos (1955) and Manning & Reid (1956) confirmed these findings. Further, Westphal (1951) reported that different brands of methylene blue gave different results; this was later confirmed by Grönroos (1955), but Beverley & Beattie (1952) contradicted it. Since methylene blue is known to hydrolyse readily in alkaline solutions, it was considered useful to study the indicator system in detail, with special reference to the lower homologues of methylene blue.

MATERIALS AND METHODS

The dye test was performed in the following manner. The positive sera used were all inactivated for 30 min. in a water-bath at 56° C. Fourfold serial dilutions were carried out up to an original dilution of 1:1024 using 0·85 % saline as the diluent. The accessory factor serum was thawed just before use. The peritoneal exudate was obtained from 6- to 8-week-old albino mice which had been infected with the RH strain of *Toxoplasma* 3 days previously from mice which themselves harboured 3-day-old infections. Only peritoneal exudates showing volumes between 0·5 and 1·0 ml. were preliminarily selected. To the peritoneal exudate from each mouse was immediately added 0·1 ml. of a 1:500 solution of heparin in saline. These suspensions were then examined under the microscope to confirm that they contained sufficient numbers of *Toxoplasma*. Only those showing 100 parasites, or

more, per high-power field were finally selected for the tests. To 2 volumes of the test serum dilutions were added 4 volumes of the accessory factor serum and 1 volume of the *Toxoplasma* suspension. An exudate control comprising 2 volumes of saline, 4 volumes of the accessory factor serum and 1 volume of the peritoneal exudate was used with every exudate. This also served as a negative control. In addition, a known positive control was set up with each batch of tests. The tests were carried out in duplicate, one for the indicator system under study and one for use with the standard indicator. The racks containing the tubes were shaken lightly to mix the contents and were incubated for 45 min. in a water bath at 37° C. At the end of this period, the racks were taken out and 1 volume of the indicator system added. The standard indicator was added to all the controls. The contents were again mixed and re-incubated for a further 5 min. The results of the tests were read immediately. A batch of results was accepted only if the accessory factor serum control showed less than 5 % modification and the positive control worked faultlessly.

The positive sera (C, D and E), showing titres of 1:64 and over, were from 3 rabbits immunized with the avirulent RB25 strain of *Toxoplasma gondii*. The sera (A and B), which had a titre of 1:4, were obtained from two rabbits which were not experimentally infected with *Toxoplasma*. A few tests with eosin and phloxines were also carried out using a known human positive serum.

The standard indicator system consisted of a 0·25 % solution of methylene blue in carbonate-borate buffer at pH 11 (prepared according to the method of Sabin & Feldman, 1948) which had been matured for a week. Fresh alkaline methylene blue also functioned perfectly. The dyes used in this study belonged to three groups, namely quinone-imine, phenyl methane and xanthene dyes. The quinone-imine dyes used were thionine (920), azure C (MacNeal), azure A (MacNeal), azure B (MacNeal), methylene blue (922) and toluidine blue (925) belonging to the subgroup thiazine dyes; brilliant cresyl blue (877), Nile blue (913) and celestine blue (900) belonging to the subgroup oxazine dyes; and neutral red (825) belonging to the subgroup azine dyes. The phenyl methane dye used was methyl green (684) and the xanthene dyes were eosin Y (768), phloxine (778), phloxine B (774) and pyronine Y (739). The numbers given within the brackets give the old colour index number for the purpose of easy recognition of the dye concerned (see Conn, 1953). The dyes were those supplied by Messrs George T. Gurr Ltd., London. For the test dye solutions, 0·25 % solutions of these dyes in physiological saline or carbonate-borate buffer at pH 11 (as recommended by Sabin & Feldman, 1948) were prepared just before they were required. As thionine and azure C were only sparingly soluble in water, a saturated alcoholic solution was first prepared. To 3 ml. of this solution were added 10 ml. of saline or carbonate-borate buffer as required. Two volumes of saline and alkaline thionine and azure C were added to the tests, instead of the usual 1 volume, to give sufficient concentration of the dye. The phloxines and the eosin were used in 2·5 % concentrations. Methyl green and neutral red were also tried in 0·5 % concentrations.

Known positive and negative dye tests, after the addition of alkaline eosin, alkaline phloxines and neutral toluidine blue, were centrifuged at 2000 r.p.m. for

10 min. and washed with alkaline buffer. The sediments were then examined for stained *Toxoplasma*.

RESULTS

The results are summarized in Table 1. All titres are given in the original dilutions. Thionine, azure A, azure B, toluidine blue, brilliant cresyl blue and Nile blue were found suitable for use as indicators in the dye test both as saline solutions and alkaline solutions. Neutral red was tried only as a saline solution because it turned yellow and formed a copious precipitate on the addition of the alkaline buffer.

Table 1. *Dye test results with saline and alkaline indicator systems using positive rabbit sera*

Dye	A	B	C	D	E
Thionine sal.	1:4	1:4	1:64	1:256	1:256
Thionine alk.	1:4	1:4	1:64	1:256	1:256
Azure C sal.	—	—	—	—	—
Azure C alk.	—	—	—	—	—
Azure A sal.	1:4	1:4	1:64	1:256	1:256
Azure A alk.	1:4	1:4	1:64	1:256	1:256
Azure B sal.	1:4	1:4	1:64	1:256	1:256
Azure B alk.	1:4	1:4	1:64	1:256	1:256
Toluidine blue sal.	1:4	1:4	1:64	1:256	1:256
Toluidine blue alk.	1:4	1:4	1:64	1:256	1:256
Brilliant cresyl blue sal.	1:4	1:4	1:64	1:256	1:256
Brilliant cresyl blue alk.	1:4	1:4	1:64	1:256	1:256
Nile blue sal.	1:4	1:4	1:64	1:256	1:256
Nile blue alk.	1:4	1:4	1:64	1:256	1:256
Celestine blue sal.	—	—	—	—	—
Celestine blue alk.	—	—	—	—	—
Neutral red sal.	—	—	—	—	—
Methyl green sal.	—	—	—	—	—
Methyl green alk.	—	—	—	—	—
Eosin Y sal.	—	—	—	—	—
Eosin Y alk.	—	—	—	—	—
Phloxine sal.	—	—	—	—	—
Phloxine alk.	—	—	—	—	—
Phloxine B sal.	—	—	—	—	—
Phloxine B alk.	—	—	—	—	—
Pyronine Y sal.	—	—	—	—	—
Pyronine Y alk.	—	—	—	—	—
Methylene blue alk.	1:4	1:4	1:64	1:256	1:256

sal. = saline solution; alk. = alkaline solution.

Even the saline solution turned yellow when added to the serum–accessory factor–exudate mixture. Saline and alkaline solutions of azure C formed only a very dilute solution when added to the reaction tubes even when double volumes were used. The staining of unmodified *Toxoplasma* was consequently very faint and differentiation of these from the modified ones on this ground was difficult. The alkaline methylene-blue solution worked well from the time of preparation until about 3 weeks, when it no longer functioned. On the other hand, the neutral toluidine blue and azure B solutions continued to give satisfactory results for 5 and 7 months respectively. The solutions were stored at room temperature throughout.

Saline and alkaline solutions of celestine blue, methyl green, eosin Y, phloxine, phloxine B and pyronine Y did not stain cytoplasm-modified or unmodified *Toxoplasma* even after a few hours' duration. The modified *Toxoplasma* appeared shrunken and shrivelled while the unmodified ones kept the normal appearance. Because of this difference the modified ones could be differentiated from the unmodified ones, although with some difficulty as the former were not very visible. The toluidine-blue-stained *Toxoplasma*, which were washed with alkaline buffer, remained stained when the sediment was examined. On the other hand, washed *Toxoplasma* sediments from positive and negative tests to which were added eosin and phloxine indicator systems remained unstained. Saline solutions of neutral red did not stain either modified or unmodified *Toxoplasma* even after a few hours' action of the dye. Similarly, 0·5 % solutions of methyl green and neutral red had no effect. The dye tests carried out with human serum, using eosin and phloxines as indicators, functioned in the same manner as those carried out with rabbit sera.

DISCUSSION

Sabin & Feldman (1948) observed that thionine, toluidine blue, methylene blue (thiazines), brilliant cresyl blue (oxazine) and neutral red (amino azine) stained the cytoplasm of normal *Toxoplasma*. In aqueous solutions these dyes acted slowly and required from $\frac{1}{2}$ to 2 hr. for optimum staining. On adjustment of the pH to 11, using a carbonate-borate buffer, the staining became instantaneous. On the other hand, phloxine (xanthene) in 2·5 % concentrations, in either aqueous or alkaline solutions, was observed to stain the cytoplasm of modified *Toxoplasma* but not the normal organisms. Commercial basic fuchsin (rosaniline) stained modified *Toxoplasma* more readily than normal *Toxoplasma*. In the presence of phenol (as in Ziehl–Neelsen stain) both types of *Toxoplasma* were stained rapidly and well. Crystal violet (rosaniline) stained normal more readily than modified *Toxoplasma*. Acid fuchsin (rosaniline), brilliant vital red, Congo red, trypan red (azo dyes) and sodium 2, 6-dichloro-benzenone-indophenol failed to stain either cytoplasm-modified or unmodified *Toxoplasma*. Fehrle (1955) observed vital staining of normal *Toxoplasma* using 0·5 % aqueous or alcoholic solutions, depending on their solubilities, of the following dyes diluted 1 : 3 with the appropriate buffer: Methylenblau, Toluidinblau, Thionin, Methylgrün (thiazines), Nilblausulfat, Brillantkresylblau (oxazine), Safranin, Neutralrot (azines), Auromin (auromine), Methylviolett, Malachitgrün, Fuchsin, Brillantgrün, Kristalviolett (triphenylmethane derivatives), Viktoriablau, Nachtblau (diphenylnaphthylmethane dyes), Akridinrot, Pyronin (xanthones), Akridinorange, Akridingelb, Coriphosphin, Diamantphosphin, Brillantphosphin (acridine dyes), Chrysoidin, Janusgrün and Bismarckbraun (azo dyes). The reactions of the optimum staining solutions were found to vary with each of the dyes but for most of them the optimum value was above pH 7. The intensity of staining did not vary with the concentration of the staining solution. On the other hand, modified *Toxoplasma* were stained by 2·5 % aqueous solutions of the following dyes: Brillantreinblau (auromine), Patentblau B, Cyanol, Formylviolett S 4 B (triphenylmethane dyes), Brillantindocyanin 6 B (triarylmethane dye), Phloxin, Eosin, Echtsäureviolett A 2 R, Bengale

Rose, Rhodamin (xanthene dyes), Kongorot, Trypanblau, Trypanrot, Ponceau, Bordeauxrot, Viktoriaviolett, Sulforhodamin B, Brillant Crocein MOOL, Chromotrop 2R, Alkaliechtgrün, Supranolrot B and Geranin (azo dyes). The dyes Säureviolett, Säurefuchsin, Wasserblau (triphenylmethane dyes), Alizarinrot (anthraquinone) and Naphtholgrün (nitroso dye) did not stain modified *Toxoplasma*. Of the basic dyes which were suitable for staining normal *Toxoplasma*, alkaline solutions of Toluidinblau, Kristallviolett, Methylviolett, Thionin, Brillantkresylblau and Methylgrün and neutral solutions of Neutralrot, Akridinorange and Coriphosphin were tried out in the dye test, with methylene blue for comparison, and were found suitable for use as indicators of unmodified *Toxoplasma*. Similarly, Fehrle (1955) used aqueous solutions of Formylviolett S4B, Patentblau B, Kongorot, Brillantcrocein MOOL, Supranolbrillantrot B, Viktoriaviolett, Chromotrop 2R, Trypanblau, Sulforhodamin B, Echtsäureviolett A2R, Eosin, Phloxin, Brillantindocyanin 6B and Brillantreinblau in dye tests and found them serviceable as indicators of modified *Toxoplasma*.

Although Sabin & Feldman (1948) and Fehrle (1955) claimed to have stained modified *Toxoplasma* with phloxine, all attempts to do so in this study were failures. The claim of Fehrle (1955) that eosin also stained *Toxoplasma* after the action of the dye test antibodies could not be substantiated under the experimental conditions adopted here. One possible explanation of this behaviour is that 'phloxines' and 'eosin' different from those used in this study may have been used by Sabin & Feldman (1948) and Fehrle (1955). Additional support for this view comes from the fact that 2 dyes, Congo red and trypan red which Sabin & Feldman (1948) claimed did not stain modified or unmodified *Toxoplasma*, were found by Fehrle (1955) to stain modified *Toxoplasma*. Another explanation suggested by Westphal, Bimmer & Palm (1957), on the basis of the observation of Braunsteiner, Pakesch & Thalhammer (1957) who reported that in modified *Toxoplasma* there was a complete breakdown of the cytoplasmic organelles which were replaced by a structureless mass, was that the staining observed might be due to staining of the cell membrane. As against this view, no staining with phloxines and eosin was observed in washed dye test *Toxoplasma* which had been subjected to the action of these dyes. In the present study neutral red did not stain normal *Toxoplasma*, as claimed by Sabin & Feldman (1948) and Fehrle (1955), even after a few hours' action of the dye. Methyl green also behaved similarly, in opposition to the claim of Fehrle (1955). As in the previous instance the only explanation possible is that different dyes were being used. This possibility is strengthened by the fact that the Methylgrün of Fehrle (1955) was a thiazine dye, whereas the methyl green used in this study is a triphenyl methane dye.

Cathie & Dudgeon (1949) found that the dye solution improved during ageing, and that it retained the ability to stain for 14 days. This was partly confirmed by Grönroos (1955), who found that certain brands of dyes improved on storage at room temperature and all the brands of dyes he examined tolerated storage at room temperature well. Manning & Reid (1956) also found that the dye solutions improved on storage and could be kept without deterioration for 2–3 weeks. On the other hand, Westphal (1951) found significant differences in the titre using

dyes of different origin. He found that the samples of dyes sent by Sabin and by Feldman, although they were powdery and crystalline respectively, gave identical results, which were higher than those obtained using his samples of dyes. Therefore he concluded that the colloidal structure of the dye had no effect, but the source of origin of the dye was important in relation to the titre. His observations were confirmed by Grönroos (1955), who found that storage tended to equalize these differences. On the other hand, Beverley & Beattie (1952), Jacobs & Cook (1954) and Frenkel & Jacobs (1958) found that there were no differences in the reaction which could be ascribed to different origins of the methylene blue. Contrary to the observation of Westphal (1951), Westphal *et al.* (1957) reported that Neumethylenblau N and Neumethylenblau D232 when freshly prepared failed to stain normal *Toxoplasma* but, after storage, these dyes acted on the *Toxoplasma* as expected.

It is generally known that methylene blue readily undergoes hydrolysis in alkaline solutions, giving a mixture of its lower homologues. According to Conn (1953) there are indications that pure methylene blue is a relatively poor histological stain. Holmes (1927) found that practically all commercial samples of methylene blue contained appreciable proportions of azure B. One sample even contained as much as 10% of azure B (Holmes, 1928). Therefore some of these differences may be attributed to the different percentages of the lower homologues present in different samples of commercial methylene blue. After some time in alkaline solutions, the 'inferior' methylene blue adjusts itself by hydrolysis. This view is further supported by the results of the present investigation. Most of the dyes found suitable for use in saline solutions were lower homologues of methylene blue or related compounds which have a common starting material during manufacture and hence may be expected to contain one another as impurities.

The present study established that the basic dyes thionine, azure A, azure B. toluidine blue and brilliant cresyl blue stained normal *Toxoplasma* both in saline and in alkaline solutions. Methyl green, pyronine Y and celestine blue did not stain either modified or unmodified *Toxoplasma* in alkaline or neutral solutions. It was also found that azure B and toluidine blue in saline solutions could be kept at least 5 months, if not more, at room temperature without deterioration. Perhaps they could be stored indefinitely in the refrigerator. Hence it is practicable to prepare a stock solution of either of these dyes and to use it when required instead of preparing more frequently the alkaline solutions.

Finally it could be concluded that the chemical configuration of the dye is not the sole criterion for use as the indicator in the Sabin–Feldman test, as, among closely related dyes, some were found which did not give the reaction while more distantly related dyes gave the reaction. Since none of these dyes is 100% pure and as they are all manufactured from the same basic material, there is a remote possibility that one of them (or another unsuspected dye) may be common to all and act as the active constituent. However, the observations of Grönroos (1955) and Westphal *et al.* (1957) and some of the present observations do not support this view. Kulasiri & Dasgupta (1959) substantiated by means of cytochemical tests the suggestion of Bringmann & Holz (1953) that the disappearance of

ribonucleic acid during the action of the dye test antibodies was apparently the basis of this reaction. The possibility that only dyes which combine with this substance under vital conditions would be suitable for use in the test is suggested by these findings.

SUMMARY

Azure A, azure B and Nile blue in both neutral and alkaline solutions were found to be suitable for use as indicator dyes in the Sabin–Feldman test. Toluidine blue, brilliant cresyl blue and thionine were also suitable as neutral solutions. Azure C, phloxine, phloxine B, neutral red, methyl green, pyronine Y, eosin and celestine blue were found to be unsuitable. Azure B and toluidine blue in saline solutions were serviceable up to 5 months after preparation. The significance of these findings is discussed.

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A re-description of the trematode *Calicotyle stossichii* Braun, 1899, with an account of *Calicotyle palombi* sp.nov.

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(*Received* 2 *March* 1959)

This account is based upon the study of forty monogenetic trematodes belonging to the genus *Calicotyle* Diesing, 1850, found in the following hosts, locality and habitats:

Host	Locality	No. of parasites in rectal glands	No. of parasites in recta and cloacae
Mustelus mustelus	Mediterranean (Sète)	17	8
M. canis	Mediterranean (Sète)	2	12
Centrophorus uyato	Mediterranean (Sète)	—	1

In order to make a preliminary study of their anatomy, the specimens were detached from the hosts and examined alive in a drop of sea water on a slide. They were then fixed in Bouin-Hollande and either stained with alum carmine for the preparation of whole mounts or sectioned in three planes, transverse, saggittal and facial, before staining with Erhlich's haematoxylin and eosin. Observations on the material have shown that it includes two species of *Calicotyle*, namely *C. stossichii*, first described by Braun (1899), and a new, closely related species which has been named *C. palombi*. The two species differ in their habitat, in the shape of the body, in the distribution of the yolk glands, in the shape of the ovary and in the size of the hooks of the posterior adhesive organ (Figs. 1–11). *C. stossichii* was briefly re-described by Palombi (1949), but a further account is given below as our knowledge of the species is inadequate. This is followed by a brief description of the specific characters of *C. palombi* sp.nov. A discussion of the tendency towards the development of endoparasitism in the Calicotylidae is also given together with a key to the species in the family.

Calicotyle stossichii Braun, 1899

External features. The body is bright red in colour, parallel sided (Fig. 1) and measures up to 10·0 mm. in length and 3·5 mm. in maximum breadth. The mouth is ventral, subterminal and surrounded by a weakly developed oral sucker which is approximately 0·60 mm. in diameter. The genital atrium lies on the median line a short distance behind the bifurcation of the intestine. The two vaginal openings (Figs. 1, 9) are ventral, almost level with the genital atrium and lying close to the

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inner sides of the intestinal caeca. The adhesive organ (Fig. 1), which is armed with two hooks, is transversely oval in outline and its anterior border overlaps the posterior end of the body. The margin of the adhesive organ is permanently curved

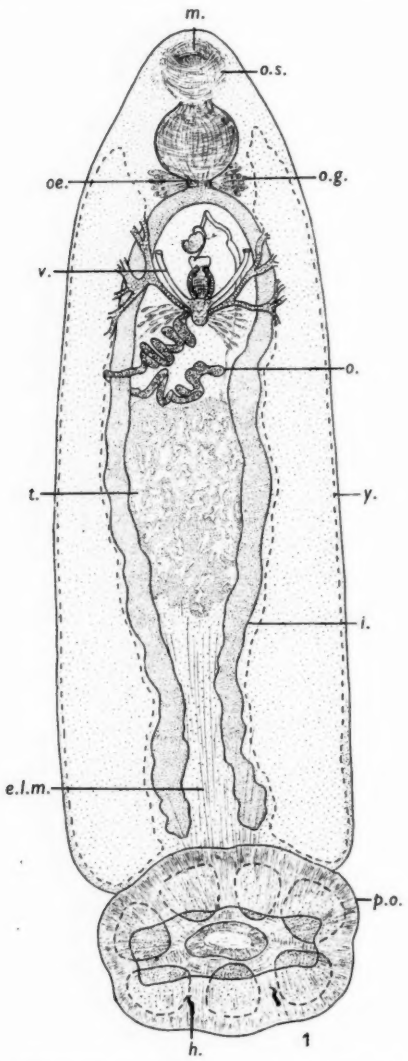


Fig. 1. *Calicotyle stossichii* Braun, 1899. Ventral view.

ventrally towards the centre of the organ, thus forming a very shallow bowl-shaped structure. This incurved margin conceals, almost completely, seven peripheral loculi. The paired hooks (Fig. 6) are weakly developed compared with those of *Calicotyle kroyeri* Diesing, 1850. In this latter species, found by the writers in

various members of the genus *Raja*, each hook is about 0·35–0·38 mm. long, but in *C. stossichii* they never exceed 0·12 mm.

Integument and musculature. The form of the integument and musculature of *C. stossichii* agrees with the accounts given by Wierzejski (1877) and Goto (1894)

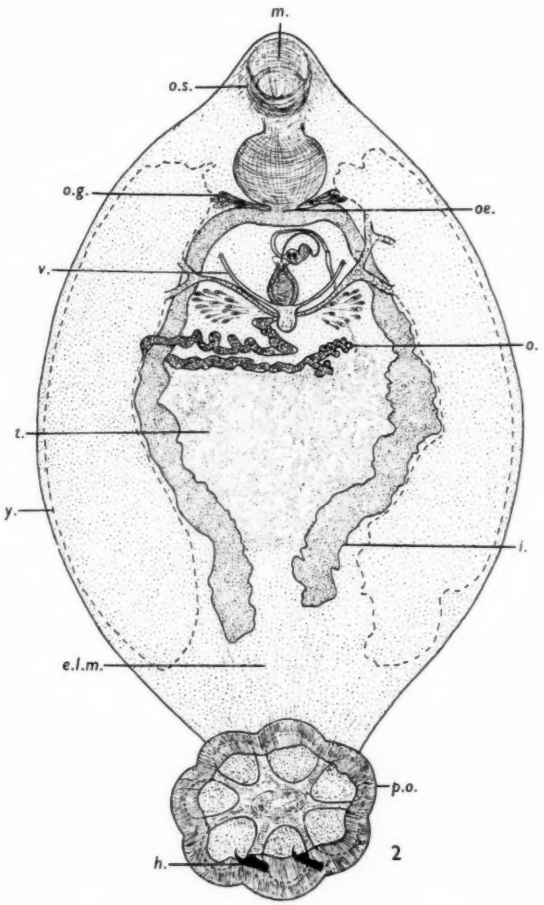
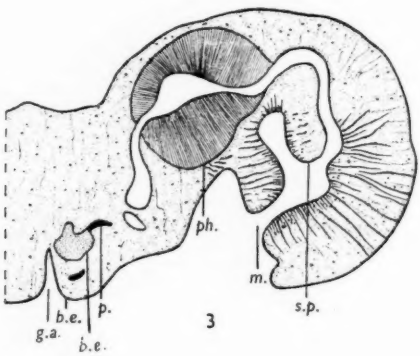


Fig. 2. *Calicotyle palombi* sp.nov. Ventral view.

for other members of the genus *Calicotyle*. It is, therefore, considered unnecessary to give another description here.

Alimentary system. The mouth opens through the oral sucker into a very short thin-walled pre-pharynx (Figs. 1, 4 and 9) which leads to the spherical muscular pharynx. The structure of the oral sucker is interesting in view of the account of *Calicotyle mitsukurii* by Goto (1894) and a comment on *C. stossichii* by Brinkmann (1952). Goto stated that the oral sucker is 'a deep invagination of the ventral side of the body just behind the mouth'. His figure, 8, plate XIX, of the oral

sucker resembles closely that of *C. stossichii* as seen from serial sections (Fig. 4). Brinkmann (1952), however, refers to a 'sucker pad' within the oral sucker of *C. stossichii* and suggests that it may be absent in *C. kroyeri*. The present investigation confirms the presence of a prominent structure, presumably a 'sucker pad', in *C. stossichii*, but serial sagittal sections of *C. kroyeri* also show the presence of a similar, though relatively smaller structure (Fig. 3). The pharynx is followed by a very short oesophagus on either side of which is a group of gland cells opening into its lumen. Immediately behind these two groups of gland cells the oesophagus bifurcates into simple unbranched intestinal caeca. These run almost parallel with



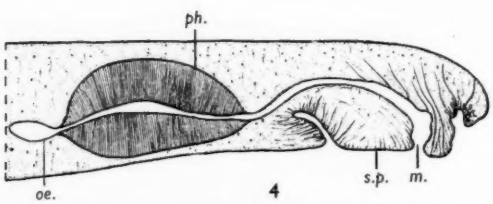


Fig. 3. *Calicotyle kroyeri* Diesing, 1850. Median sagittal section through oral sucker.

Fig. 4. *C. stossichii* Braun, 1899. Median sagittal section through oral sucker.

each other and with the lateral margins of the body as far as the anterior border of the posterior adhesive organ. The caeca have a diameter of about 0·20 mm. and show a tendency towards sacculation.

Nervous system. The nervous system will not be described here as it appears to resemble that of *Calicotyle mitsukurii* described by Goto (1894).

Reproductive system. The male genital pore opens anteriorly and the female pore posteriorly into a deep genital atrium situated a short distance behind the bifurcation of the oesophagus (Figs. 9, 10).

(*a*) *Male genitalia.* The vas deferens (Figs. 9, 10) arises dorsally from the left anterior border of the testis and runs forwards almost parallel and slightly to the inner side of the left intestinal limb. Near the left vaginal opening it curves gently towards the middle line to form the vesicula seminalis. The vesicula seminalis

is continuous with the bulbus ejaculatorius whose slightly dilated proximal end contains two small cavities. Strongly developed muscle bands are attached to the junction of the vesicula seminalis and bulbus ejaculatorius (Figs. 7, 9). The distal end of the bulbus ejaculatorius is continuous with a thin flexible penis which is about 0·48 mm. long and 0·0072 mm. in diameter. The penis opens to the exterior at the apex of a well-developed lobe (Fig. 10) which projects into the anterior end of the genital atrium. The penis can be protruded to the exterior through the opening on the penis lobe or withdrawn into the cavity of the penis-sheath.

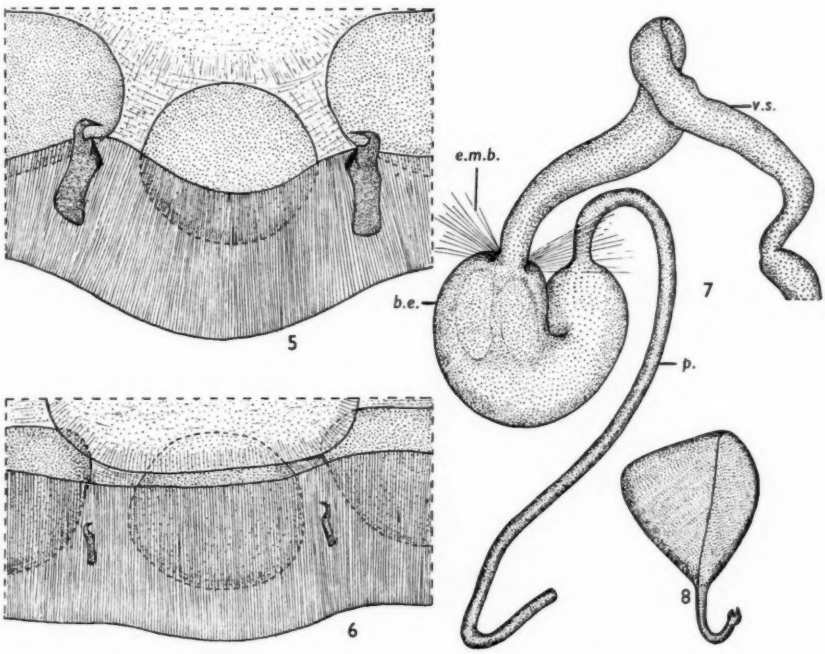


Fig. 5. *Calicotyle palombi* sp.nov. Hooks of the posterior adhesive organ.

Fig. 6. *C. stossichii* Braun, 1899. Hooks of the posterior adhesive organ.

Fig. 7. *C. stossichii* Braun, 1899. Vesicula seminalis, bulbus ejaculatorius and penis.

Fig. 8. *C. stossichii*, Braun, 1899. Egg.

(*b*) *Female genitalia.* The ovary is tubular, convoluted and roughly of equal diameter throughout its length (Figs. 9, 10). The distal part of the ovary is slightly swollen and shows a tendency towards lobation. The ovary runs posteriorly at first and then extends antero-laterally from the left anterior border of the testis up to and around the right intestinal limb and returns towards the middle line of the body. Here it narrows to form the oviduct which leads into the ootype. This latter structure is in the middle line slightly in front of and at right angles to the oviduct, being continuous posteriorly with the yolk reservoir. Two groups of gland cells, presumably Mehlis's glands, are situated one on either side of the ootype which

receives the contents of the gland cells. The distal portion of the ootype is very muscular, acting probably as a mould for the egg. Anteriorly the ootype merges into a very short thin-walled uterus which opens into the genital atrium.

The yolk glands are very much branched and situated laterally, on each side of the body. They occupy the areas between the intestinal caeca and the lateral

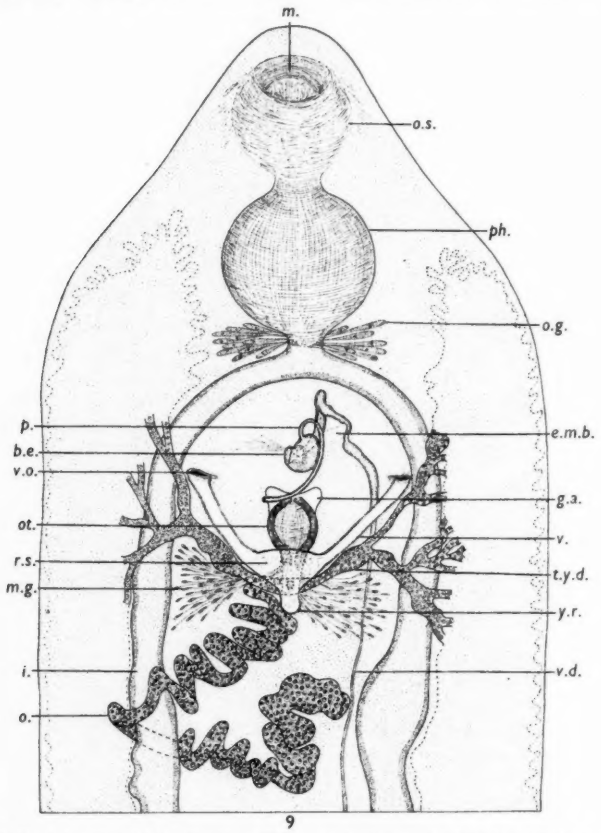
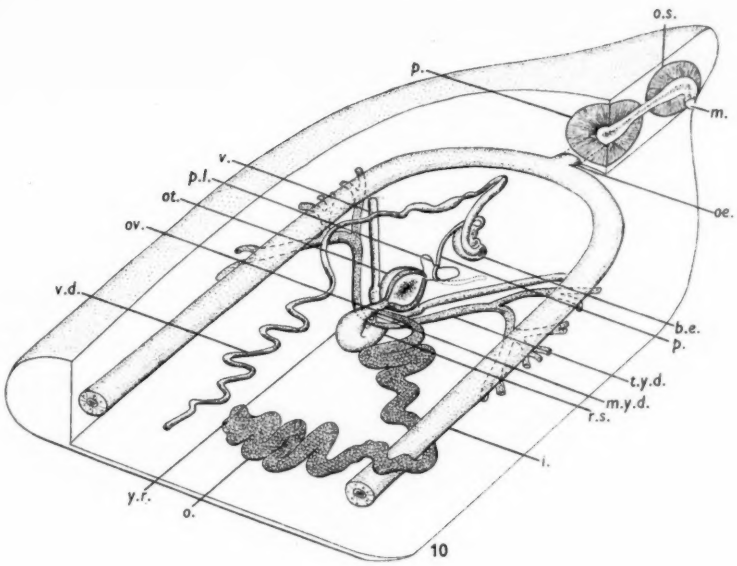


Fig. 9. *Calicotyle stossichii* Braun, 1899. Anterior end showing male and female genitalia, ventral view.

margins of the body (Figs. 1, 9) and extend from the level of the anterior border of the pharynx to the posterior end of the animal Near the level of the genital atrium three ducts leave each yolk gland and unite to form a main collecting vessel which runs posteriorly towards the middle line, where both meet to form a short median yolk duct. This latter opens into the ventral side of the yolk reservoir. The two vaginae which are surrounded by glandular cells form a distinct V. They open, one on each side, into the mid-ventrally situated receptaculum seminis which lies below the yolk reservoir and is continuous with it. The eggs are tetrahedral in shape with a short filament which possesses a swollen and bifurcated tip (Fig. 8). They are about 0·17 mm. long.



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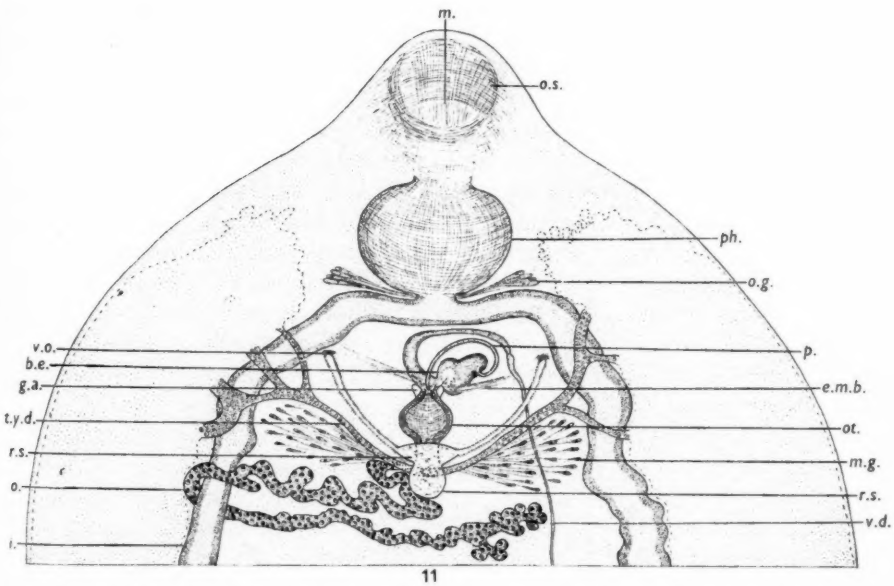

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Fig. 10. *Calicotyle stossichii* Braun, 1899. Diagram of male and female genitalia, dorsal view.

Fig. 11. *Calicotyle palombi* sp.nov. Anterior end showing male and female genitalia, ventral view

Calicotyle palombi sp.nov.

It is thought unnecessary to give a detailed description of *C. palombi* sp.nov. as it closely resembles *C. stossichii*. It differs from the latter species, however, in the following characters, which are at present considered to be of sufficient importance to segregate the two species.

The body is pale red in colour and almost oval in shape (Fig. 2). The specimens are up to 12·0 mm. long and 6·0 mm. broad across the middle of the body. The paired hooks of the posterior adhesive organ (Figs. 2, 5) are relatively much larger, each being 0·28–0·31 mm. long. The posterior ends of the intestinal caeca curve inwards towards the middle line and do not extend as far as the anterior border of the adhesive organ (Fig. 2). The penis is slightly longer than that of *C. stossichii*, being 0·50 mm. long. The distal end of the ovary is distinctly lobed, the number of lobes varying from 6 to 9 (Fig. 11). The yolk glands terminate posteriorly near the ends of the intestinal caeca (Fig. 2). *C. palombi* sp.nov. inhabits the cloaca or rectum of *Mustelus mustelus* and *M. canis* and has not been found in the rectal gland of these two hosts.

DISCUSSION

The occurrence of *Calicotyle palombi* sp.nov. and *C. stossichii* in the cloaca and rectal gland respectively of two species of the genus *Mustelus* is interesting in view of the account by Brinkmann (1952) on the tendency by members of the subfamily Calicotylinae to become endoparasites. He discussed the location within the host of seven species of *Calicotyle* and one of *Dictyocotyle* and arrived at the conclusion that it is not surprising to find *D. coeliaca* in the coelom of species of the genus *Raja* as some species of *Calicotyle* occupy intermediate habitats. *C. inermis* Woolcock, 1936, for example, dwells in the oviduct of *Pristiophorus cirratus*. It is possible that *Calicotyle inermis* and *C. stossichii* have arisen from a cloaca-dwelling form, like *C. palombi* sp.nov. In *C. inermis* there are no hooks on the posterior adhesive organ, and in *C. stossichii* they are very reduced in size as compared with those of *C. palombi* sp.nov. The tendency towards the loss of hooks by *C. stossichii* may have been the result of migration into the internal ecological niche which it occupies. The species of *Calicotyle* which inhabit the cloaca of elasmobranchs possess strongly developed hooks which are probably essential as organs of attachment in this particular habitat.

C. palombi sp.nov. has been erected as a new species, although there has been some recent controversy (Brinkmann, 1952; Dawes, 1958; Williams, 1958) on speciation within the subfamily Calicotylinae. It may, ultimately, become a synonym of *C. stossichii*, but the acceptance of two species at present is supported by the absence of intermediate forms. The two species differ invariably in at least six characters of their morphology or ecology. It is considered unnecessary to give a detailed discussion here of the systematics of all species of *Calicotyle* and *Dictyocotyle* as recent accounts are available. Instead, a key to the species belonging to these two genera is given below. This is based mainly on an investigation by the writers into five of the species and on descriptions given by Brinkmann (1940, 1952) and Llewellyn & Green (1957).

Key to genera

Posterior adhesive organ with numerous irregularly distributed shallow loculi. *Dictyocotyle* Nybelin, 1941

Posterior adhesive organ with seven peripheral loculi. *Calicotyle* Diesing, 1850

Specific characters of Dictyocotyle coeliaca

Posterior adhesive organ without hooks; penis 2·25–2·75 mm. long; ovary lobed; coelom of *Raja radiata* Donovan and *R. naevus* Muller & Henle.

Key to species of Calicotyle

1. Posterior adhesive organ without hooks: penis 1·0–1·8 mm. long; oviduct of *Pristiophorus cirratus.* *Calicotyle inermis* Woolcock, 1936
2. Posterior adhesive organ with hooks. **3**
3. Yolk glands do not extend to posterior end of body. **5–6**
4. Yolk glands extend to posterior end of body. **7–11**
5. Ovary distinctly lobed; penis about 0·50 mm. long; hooks about 0·3 mm long; rectum and cloaca of *Mustelus mustelus* and *M. canis* *Calicotyle palombi* sp.nov.
6. Ovary not lobed; penis 1·0–1·50 mm. long; hooks about 0·38 mm. long; rectum, rectal gland and cloaca of *Chimaera monstrosa.* *C. affinis* Scott, 1911
7. Ovary slightly lobed; penis about 0·48 mm. long; hooks about 0·12 mm. long; rectal gland of *Mustelus mustelus* and *M. canis.* *C. stossichii* Braun, 1899
8. Penis about 0·36 mm. long; hooks about 0·38 mm. long; cloaca of various species of the genus *Raja.* *C. kroyeri* Diesing, 1850
9. Penis about 0·40 mm. long; hooks about 0·34 mm. long; on *Trygonorhina fasciata.* *C. australis* Johnston, 1934
10. Penis about 0·59 mm. long; hooks about 0·56 mm. long; cloaca of *Rhina* sp. *C. mitsukurii* Goto, 1895
11. Intestine of *Raja microps* Gunther. *C. macrocotyle* Cordero, 1944

One of us (H. H. W.) has received generous financial aid from the Department of Scientific and Industrial Research.

KEY TO LETTERING OF TEXT FIGURES

b.e.	bulbus ejaculatorius	*ov.*	oviduct
e.l.m.	extrinsic longitudinal muscles	*p.*	penis
e.m.b.	extrinsic muscle bands of the bulbus ejaculatorius	*ph.*	pharynx
		p.l.	penis lobe
g.a.	genital atrium	*p.o.*	posterior adhesive organ
h.	hook	*r.s.*	receptaculum seminalis
i.	intestine	*s.p.*	'sucker pad'
m.	mouth	*t.*	testis
m.g.	Mehlis' gland	*t.y.d.*	transverse yolk duct
m.y.d.	median yolk duct	*v.*	vagina
o.	ovary	*v.d.*	vas deferens
oe.	oesophagus	*v.o.*	vaginal opening
o.g.	oesophageal glands	*v.s.*	vesicula seminalis
o.s.	oral sucker	*y.*	yolk gland
ot.	ootype	*y.r.*	yolk reservoir

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Note added in proof

Soon after this paper was submitted for publication Llewellyn (1959) and Dawes & Griffiths (1959) expressed further opinions on *Dictyocotyle* and *Calicotyle* but, at present, there are no reasons to change our views, which appear to agree with those of Llewellyn.

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Parasitology (1960), **50**, 31–49
With 5 text-figures
Printed in Great Britain

Ecological studies on the development of the pasture stages of *Nematodirus battus* and *N. filicollis*, nematode parasites of sheep

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(*Received* 9 *March* 1959)

In a previous publication (Thomas, 1960*a*) the life histories of *Nematodirus battus* and *N. filicollis* were described. It was shown experimentally that the life history could be completed in 5–6 weeks under optimal conditions. Larval development took 3–4 weeks and the prepatent period was 2–3 weeks. It was noted, however, that the infective larvae of these species did not normally hatch when development was complete, although activity could be stimulated by rupture of the egg membranes. This phenomenon did not occur in the related species *N. helvetianus* (Herlich, 1954) and *N. spathiger* (Kates & Turner, 1955), in which hatching readily occurred at 20–28° C.

In further work (Thomas 1960*b*), the seasonal incidence of these two species was investigated, and it was found that the life history was exceptional in that 12 months was required for its completion, only one generation being produced each year. Lambs became infected in spring, and the bulk of egg deposition occurred in late spring–early summer. However, no larval activity was detected until the following spring, and it was suggested that the larvae remained dormant throughout the summer and winter. To explain this long delay in the completion of the life history it was suggested that under natural conditions some special factor might be required for the completion of larval development and hatching of the infective larvae.

The aim of the present work was to provide detailed information of the development of the eggs and larvae of *N. battus* and *N. filicollis* under natural conditions, and to investigate the nature of the stimulus to hatching of the infective larvae.

FIELD EXPERIMENTS

(1) The availability of Nematodirus infection to grazing lambs

Material and methods

A strip of permanent pasture 37 yards × 2 yards, (plot A), which had not been grazed by livestock for 5 years, was contaminated with eggs of *Nematodirus*

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species by grazing it with two infected Cheviot lambs. The lambs were enclosed in a large fold-type chicken pen which was moved along the strip every few days between 24 May and 21 June 1955. The mean faecal egg counts for the two lambs during the 4 weeks grazing were 110 eggs pre gramme (e.p.g.) for *N. filicollis* and 300 e.p.g. for *N. battus*. The faecal output of the two lambs was estimated by collection of faeces from an enclosed concrete floor for 24 hr. at the beginning of the experiment; it was found to be 400 g. During the 29 days grazing, therefore, approximately 1,300,000 *N. filicollis* and 3,500,000 *N. battus* eggs were deposited on an area of 74 sq. yds., or 17,500 *N. filicollis* eggs and 47,000 *N. battus* eggs per sq.yd.

Table 1. *Mean maximum egg count and total worm burden of pairs of grazing lambs*

	No. of days before eggs appeared		Maximum eggs per gramme of faeces		Total worm count 3 weeks after leaving plot A	
Dates grazed	*N. battus*	*N. filicollis*	*N. battus*	*N. filicollis*	*N. battus*	*N. filicollis*
1955						
2–12 July	None present		Nil	Nil	Nil	Nil
21–26 July	None present		Nil	Nil	Nil	Nil
8–11 Aug.	None present		Nil	Nil	Nil	Nil
24 Sept.–1 Oct.	None present		Nil	Nil	Nil	Nil
1956						
20–27 April	18	22	100	25	240	60
30 April–6 May	18	23	125	25	370	40
9–15 May	15	19	225	25	460	75
18–24 May	15	24	125	50	220	75
4–9 June	17	20	75	50	420	410
18–23 June	16	19	100	175	570	570
15–20 Oct.	—	17	—	50	—	40

From 2 July to 1 October 1955, and again from 20 April to 23 June 1956, the plot was grazed by pairs of worm-free lambs. The lambs used in the 1955 grazing were 4–6 months old and those used in the 1956 grazing 2–3 months old. Faecal samples were taken from the grazing lambs daily, and the lambs were slaughtered 3 weeks later so that total worm counts could be done. The results (Table 1) show that no pick-up of infection occurred during the period 2 July–1 October 1955, but that a considerable infection was picked up during the period 20 April–23 June 1956. Thus no available infective larvae were present on this plot during the whole of the summer following its contamination. A single pair of lambs grazed the plot during October 1956 and developed a small *N. filicollis* burden, which indicated that a low level of infection with this species was still present, although *N. battus* had apparently disappeared.

The presence of larvae on the plot was also followed by grass-sampling at weekly or bi-weekly intervals, the method used being that of Michel & Parfitt (1955). Larvae of *N. filicollis* were first recovered at the end of October 1955; small numbers of them were present throughout the winter and their numbers increased during the following spring and early summer. Very small numbers of *N. battus* larvae were recovered in November 1955; but after that date none were

recovered until the end of March 1956, when a sudden increase occurred, the numbers reaching a maximum of 900 larvae/lb. in early May; they then declined to a low level in July (Fig. 1).

Thus the sampling results confirm the grazing results, in that infective larvae were not present in a form available to the grazing animal during the summer and early autumn following contamination of the plot. Unfortunately, after

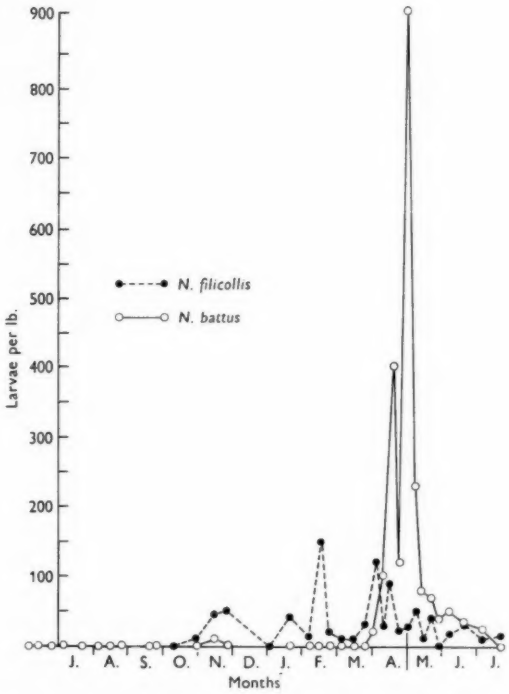

Fig. 1. Recovery of *Nematodirus* larvae from plot A, 1955–6.

October 1955, no lambs were available for grazing during the period when *N. filicollis* larvae were first recovered from herbage samples. The lambs grazing during the peak period of larval activity did not show the heavy infections which might have been expected from the high larval counts, but this may be due at least in part to the fact that the lambs only grazed for 5–6 days on the contaminated plot. In addition, exceptionally hot, dry weather during the peak period may have killed many larvae, and in fact there were few natural outbreaks reported during June 1956. The period betweeen exposure to infection and the appearance of eggs in the faeces varied between 12 and 20 days for *N. battus*, with an average of 17 days, and between 17 and 24 days for *N. filicollis*, with an average of 21 days. This is in agreement with the prepatent periods determined experimentally (Thomas, 1960*a*).

(2) THE DEVELOPMENT OF THE EGGS AND LARVAE ON THE PASTURE

The development and behaviour of the eggs and larval stages throughout the 10- to 12-month period normally spent on the pasture was studied in the following manner:

Perennial rye grass was sown in sterile soil in flower pots 6 in. in diameter and, after a period of growth, these pots were sunk in rows in an enclosed portion of a first-year ley pasture on the College farm at Cockle Park, Northumberland. The rows were 2 ft. apart, with 1 ft. between the pots in each row, and the pots were buried to leave a lip of approximately 1 in. above the soil surface. The grass on the pots was kept trimmed to a height of 3–4 in. At the beginning of each experiment one row of pots was contaminated with 10 g. of faeces per pot in the form of four equal pellets, while 10 g. of faeces broken down in 40 c.c. of water was distributed on each of a second row of pots. Sufficient pots were treated to allow for sampling of one pot per month from each row until the following July, and experiments were begun in May, June, July, September, October and November, 1956.

This design was based on the findings of a preliminary experiment carried out in 1955, in which several series of pots were laid out in May–July, each pot containing faeces in pellet form. Because the summer was extremely dry, the pellets did not break down as expected, and in early autumn the material was removed from the pots by earthworm activity, and the experiment had to be abandoned. In 1956, therefore, the experimental area was treated with 1–2 oz. of lead arsenate per sq.yd. to eliminate the earthworm population and to prevent the removal of the faecal material. In addition, each series was duplicated, liquid faeces being used to allow more rapid breakdown of the material.

Faeces for these experiments were obtained from heavily infected lambs in May and June and stored at 5° C. until required. Fresh material could not be used for each experiment, because faeces with a high egg count were obtainable only during May and June. Floatation egg counts were carried out on the faeces before use in each series, and control cultures were incubated to check the viability of the material,

At monthly intervals two pots were taken from each series, one containing faeces pellets and the other liquid faeces, and these were examined in the following manner. The grass was removed with scissors, leaving half an inch of grass stubs and mat, together with the faecal material. This layer was then removed, and the top half inch of soil was taken off. The next half inch of soil was examined from time to time, but contained very few eggs or larvae. The grass, mat and soil were then examined and it was found more convenient to handle them separately. The grass and mat specimens were placed in jars, covered with water, and left for 24 hr., while the soil sample was similarly treated with a 5% solution of a water-softener ('Calgon'), to increase the breakdown of the soil. The contents of each jar were then poured through two sieves of 100- and 400-mesh respectively, and well washed with water, faecal pellets being broken down by hand if necessary. The contents of the 100-mesh sieve were discarded and the contents of the 400-

mesh sieve, which retained both free larvae and eggs, were collected. Eggs and larvae were then recovered from this material by the floatation technique of Michel & Parfitt (1955), using a Clayton–Lane centrifuge. Previous experiments showed that a larval recovery of 53 % was obtained from artificially infected grass (Thomas, 1960*b*), and similar experiments showed a recovery of 28 % of larvae and 45 % of eggs from artificially infected soil samples.

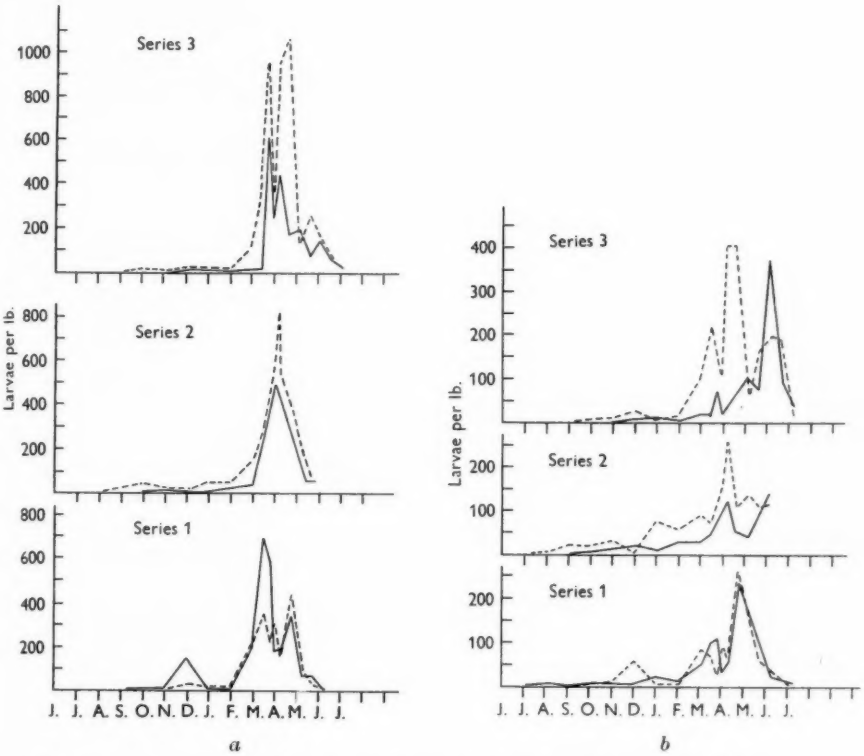

Fig. 2*a*. Recovery of *N. battus* larvae from series 1–3, 1956–7. Faecal pellets, ——; liquid faeces, – – –.

Fig. 2*b*. Recovery of *N. filicollis* larvae from series 1–3, 1956–7. Faecal pellets, ——; liquid faeces, – – –.

It was found that dead and disintegrating eggs did not normally float in salt solution, and only low recoveries of such eggs occurred. These eggs were therefore not included in the final count. The numbers of larvae and eggs recovered were recorded, together with the percentage development of the eggs. The eggs were then incubated in water at 21° C. to test the viability and hatchability of the various stages present. The larval recoveries are shown graphically in Figs. 2*a*, *b*. Air temperatures and rainfall were recorded at a weather station 400 yd. from the experimental plot, and are shown in Table 2.

In order to compare the experimental results with those obtained under completely natural conditions, an adjacent plot, (Plot B), of 5 yd. × 11 yd. was contaminated evenly with 8·5 kg. of faeces containing 110 e.p.g. of *N. filicollis* eggs and 140 e.p.g. of *N. battus* eggs during the period 24 May–13 June. This area was sampled at monthly intervals for infective larvae, and the results are shown in Fig. 3.

Table 2. *Weekly mean temperatures* (°F.) *and total weekly rainfall, Cockle Park*, 1956–7

Date	Max.	Min.	Mean	Rainfall (in.)	Date	Max.	Min.	Mean	Rainfall (in.)
5 May	57·3	39·3	48·3	0·03	5 Jan.	47·4	33·3	40·3	0·58
12	60·9	43·7	52·3	0·07	13	47·9	37·4	42·6	0·16
19	57·1	38·8	47·9	0·17	20	40·6	31·7	36·6	0·25
26	61·3	39·4	50·3	0	26	46·6	33·7	40·1	0·99
2 June	65·1	46·9	56·0	0·23	2 Feb.	47·4	33·3	40·3	0·86
9	59·3	43·6	51·4	0·24	9	48·6	35·3	41·9	0·73
16	58·6	41·7	50·1	1·66	16	43·1	31·4	37·2	1·24
23	62·9	47·1	55·0	0·19	23	41·6	27·9	34·7	0·62
30	63·3	47·4	55·3	0·17	2 Mar.	45·4	32·1	38·7	0·03
7 July	66·1	51·6	58·8	0·9	9	48	37·3	42·6	0·03
14	64·6	48·1	56·3	0·6	16	56·6	44·7	50·6	0·39
21	62	50·8	56·4	0·33	23	52	39·9	45·9	0·48
28	66·3	53·4	54·8	0·36	30	48·6	36·4	42·5	0·34
4 Aug.	60·7	48·1	54·4	1·16	6 Apr.	52·1	40·4	46·2	0·08
11	64·7	46·0	55·3	1·26	13	46·3	36	41·1	0·03
18	58·1	48·7	54·4	2·12	20	56·6	37·7	47·1	0·09
25	60·9	43·6	52·2	2·36	27	51·6	37·3	44·4	0·17
1 Sept.	56·9	41·6	49·2	2·00	4 May	53·7	39·1	46·4	0·13
8	60·1	48·7	54·4	1·87	11	48·7	35·3	42	0·51
15	62	46·9	54·4	0·35	18	57·3	41·7	49·5	1·18
22	64·9	47·6	56·2	0·07	25	53·3	39	46·1	0·15
29	63	49·6	56·3	0·18	1 June	58·2	41·1	49·6	0
6 Oct.	55·9	41·7	48·8	0·55	8	60·1	44·4	52·2	0·51
13	56·3	41·4	48·8	0·05	15	63·4	44·3	53·6	0·06
20	55·6	41	48·3	0·51	22	65·7	46·2	56	0·41
27	52·6	39·9	46·2	0·35	29	62·1	46·9	54·5	0·39
3 Nov.	49·7	34	41·8	0·76	6 July	65·9	51·3	58·6	0·58
10	52	34·3	43·1	0·18	13	64·1	53·6	58·8	0·75
17	49	31·2	40·1	0·31	20	63·6	51	57·3	1·53
24	44·2	33·7	38·9	0·19	27	64·1	53·1	58·6	0·66
1 Dec.	45·3	33·3	39·3	0·15					
8	53·9	43·4	48·6	0·08					
15	48·9	37	43·0	0·47					
22	44·9	32·1	38·5	0·28					
29	39	29·1	34·0	1·18					

Results

The results obtained from eggs in faecal pellets and from eggs in liquid faeces were similar throughout, although development was always more rapid and was completed in a higher percentage of eggs in the liquid faeces. This similarity of results was probably due to the extremely wet summer of 1956, the faecal pellets breaking down steadily over 3–4 months, as compared with 1955, when the pellets did not break down at all during the summer. The degree of development of the

eggs in faecal pellets showed more variation from month to month, being related to variations in the degree of breakdown of the pellets. Egg recovery varied from 4 to 80%, with a mean of 29%, the recovery being consistently lower in the liquid faeces, because of the greater dissemination of the eggs in these. When less than 50 eggs were recovered the results were discarded.

Series 1

The first series of pots was contaminated with faecal material on 17 May. The faeces contained 360 e.p.g. of *N. battus* eggs and 180 e.p.g. of *N. filicollis* eggs and a control culture showed 95% viability for both species.

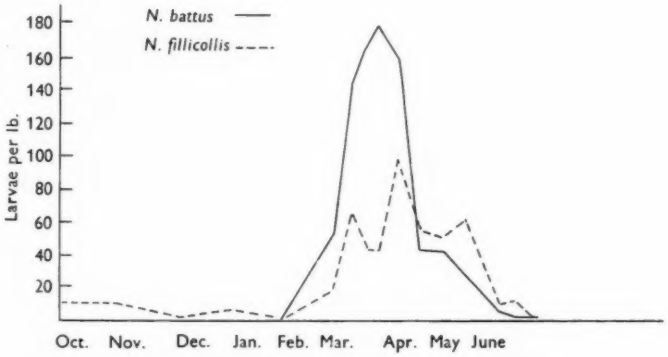

Fig. 3. Recovery of larvae from plot B, 1956–7.

N. battus. Very little development occurred in the first 2 months of exposure, probably because of the dry conditions then prevailing; but during July–September development proceeded steadily, and by September 80–90% of the eggs recovered contained fully developed third-stage larvae. A high proportion of these eggs remained fully viable throughout the winter. However, very few hatched larvae were recovered, except for a single recovery of 145 larvae in December, until the following March. During March hatching proceeded rapidly, and the larval recovery rose to a peak on 30 March. A rapid fall then followed and this was succeeded by a second peak in early May, after which date the larval recovery declined to zero in July.

N. filicollis. Development was again slow in May–June, but a small proportion of eggs had reached the third larval stage by 10 July, in contrast to *N. battus*, in which very little development occurred during this period. Development was rapid during July–September, 70–80% of the eggs having completed development by 14 August and 90–100% by 19 September. Over 90% of the eggs remained viable throughout the winter, but again very few larvae were recovered until March, with the exception of a single recovery of 59 larvae in December. Larval recovery rose in March and April but only reached a peak in May, after which date a steady decline occurred.

Series 2

The pots were contaminated with faeces on 15 June, using material from the same source as before. A control culture showed 95 % viability for both species.

N. battus. Development proceeded steadily in July–September, 50–60 % of the eggs reaching the third larval stage in the faeces pellets and 85–97 % in the liquid faeces. Of the undeveloped eggs recovered, a high proportion completed development when they were incubated at 21° C., including those recovered in May after 11 months exposure, in the pasture, and all stages showed a high percentage of viability throughout the winter. As before, very few hatched larvae were recovered until the following March, when the larval count rose rapidly to reach peak counts in April—2–4 weeks later than in Series I—and then declined rapidly to a low level in July.

N. filicollis. Development proceeded steadily in the faecal pellets, but much more rapidly in the liquid faeces, in which 90 % of the eggs completed development in 2 months. Eggs in all stages showed a high percentage viability throughout the winter. A small number of hatched larvae were recovered in September–December, increasing in January–March, and reaching a peak in April. A brief decline followed, but considerable numbers of larvae were still present in June, and in the pot contaminated with faecal pellets a second peak occurred. This was possibly due to delayed hatching of eggs which had partially developed the previous autumn and then had completed development to the infective stage in the spring.

Series 3

This series was started on 14 July, using faecal material from the same source as for Series 1 and 2. A control culture showed 94 % viability for *N. filicollis* and 93 % for *N. battus*.

N. battus. In faecal pellets egg development proceeded very slowly, 80 % showing no development after 3 months exposure, possibly because of the slow breakdown of the pellets in this series. However, considerable development appeared to occur during October–December and subsequently 40–50 % of the recovered eggs were in the third larval stage. In liquid faeces development proceeded rapidly, 80–90 % reaching the third stage by October. All stages overwintered successfully, and continued to develop in May–July. Again larval recovery was very low until March, when a rapid rise occurred, reaching a peak in April–May, and subsequently declining steadily to a low level in July.

N. filicollis. Development was slow in the faecal pellets until October after which considerable development occurred, 30–60 % of the eggs reaching the 3rd stage by December. Eggs which had not completed development in the autumn continued to develop in April–June, and these eggs apparently hatched when fully developed, for the proportion of *N. filicollis* eggs present fell from a constant 25–30 % until the end of May to only 5–10 % in June–July. In addition, the rise in larval recovery in the spring was slow, reaching a small peak in April, and then rising to a higher peak in late June. This late peak count coincides with the late rise observed in Series 2, and tends to confirm the suggestion that this second peak

is due to the delayed hatching of larvae which partially developed during the autumn but only completed development in the spring. In liquid faeces development was rapid, 97 % having completed development by October, but very few hatched larvae were recovered until March, when a rapid increase occurred, which reached a peak in late April–early May, and declined to a low level by the end of July.

Series 4

This series was begun on 19 September, with material from the same source as for the earlier series. A control culture showed 90 % viability for both species.

N. battus. Very little development occurred during the autumn, 4–5 % of the eggs reaching the morula stage and 4–5 % the first larval stage. However, the eggs recovered showed 60–88 % viability throughout the winter, and in the spring development proceeded steadily, 6 % reaching the third larval stage by July. No hatched larvae were recovered, even in June–July, when small numbers of fully developed eggs were present.

N. filicollis. Development started in a considerable proportion of the eggs in September–December, 80 % of the eggs in liquid faeces reaching the morula stage, and 3 % reaching the first larval stage. No further development occurred during the winter, but from March onwards development proceeded rapidly and in July a high proportion of the eggs had completed development to the third larval stage. No fully developed eggs were present until June, and no hatched larvae were recovered until July, when two counts of 20 and 38 larvae were recorded.

Series 5

Faecal material was set out on 14 October. The material was collected in May and contained 260 e.p.g. of *N. battus* eggs and 280 e.p.g. of *N. filicollis* eggs. A control culture showed 85 % viability for *N. filicollis* and 75 % for *N. battus*, indicating some loss of viability during storage.

N. battus. No development occurred during the winter, but a considerable proportion of the eggs remained viable and developed normally the following spring, a few having reached the third larval stage by 16 July. A single hatched larva was recovered in July.

N. filicollis. In the faecal pellets no development occurred until the spring, but in the liquid faeces a very small proportion of the eggs reached the morula stage in October–November. In the spring development proceeded rapidly and over 90 % of the eggs had completed development to the third stage by July. Hatched larvae were recovered only from the liquid faeces pot examined in July, 17 larvae being recovered. However, 100 fully developed eggs were recovered from this pot and 650 from that contaminated with faecal pellets. This, together with the results of the earlier series, suggests that, when some development occurred in the autumn, at least a proportion of the eggs hatched the following spring, when development was completed. When, however, the eggs overwintered in the undeveloped state, no hatching occurred after development in the spring.

Series 6

Faecal material from the same source as that used for Series 5 was set out on 14 November. A control culture showed 63 % viability for both species, indicating a considerable loss of viability in storage, but viable eggs showed completely normal development.

N. battus. No development occurred until March, after which development proceeded steadily, and, when sampling ceased in July, a proportion of the eggs had reached the third larval stage. Up to 60 % of the recovered eggs failed to develop when they were incubated, but this loss in viability was probably due to prolonged storage of the material before use. No hatched larvae were recovered from this series, although small numbers of fully developed eggs were present in July.

N. filicollis. Again no development occurred in the faecal pellets, but a very small proportion of the eggs in liquid faeces developed to the morula stage during the winter. From March onwards development was rapid and over 90 % of the eggs had completed development by July. The viability of the recovered eggs was lower than in the previous series, but was higher than for *N. battus.* Although large numbers of fully developed eggs were present in July, only 13 hatched larvae were recovered. This supports the suggestion that eggs which only begin development in the spring do not hatch during the summer, and possibly only hatch the following spring after overwintering for a second time as fully developed eggs.

The adjacent plot, which was contaminated with faeces in May–June, gave results closely corresponding to those of Series 1–3. Low recoveries of *N. filicollis* only were recorded from October until late February, after which the larval count for both species rose rapidly to a peak in April, and then declined steadily to a low level in July. As in Series 1–3 the *N. filicollis* peak was slightly later than that of *N. battus.* It is of interest that the *N. filicollis* count declined steadily during June, as in Series 1, and did not show the late peak counts recorded in Series 2–3. This suggests that under natural conditions egg development is completed during the summer, and that delayed development and hatching in spring, as seen in Series 2–3, does not normally occur.

(3) Laboratory experiments on the nature of the hatching stimulus

In earlier studies (Thomas, 1960*a*), it was found that, on incubation in water at 20–25° C., eggs of both *N. battus* and *N. filicollis* developed to the third larval stage, but the larvae did not hatch. The results reported in the previous section show that a similar phenomenon occurs on pasture, the infective larvae remaining dormant within the egg membranes throughout the winter. In the laboratory a number of stimuli were applied to the eggs to induce hatching, including exposure to high light intensity, ultraviolet radiation, alternate high and low temperatures. and alternate drying and moistening, without success. Hatching was induced in *N. battus* eggs by exposure to 36–38° C., but no effect was observed on *N. filicollis* eggs. Larval activity and emergence could also be stimulated by rupturing the egg membranes, and for experimental purposes larvae were hatched by shaking

the fully developed eggs in water with glass beads to rupture the membranes, this method being suggested by Gibson (private communication).

However, to be of value it was necessary that the stimulus which initiated hatching should be such that it could apply under natural conditions. Analysis of the environmental conditions suggested that temperature change was the most likely factor. Furthermore, the observation that the larvae overwintered in a dormant state and then hatched in the spring suggested that exposure to low temperatures during the winter might sensitize the larvae to the rise in temperature in the spring. To test this hypothesis the following experiments were carried out.

(*a*) *The hatching of eggs after exposure to low temperature*

Method and material

Eggs of *N. battus* and *N. filicollis* were incubated at 21° C. in Petri dishes containing water to a depth of 5 mm. When development to the third larval stage was completed, the cultures were plated at 3° and −2° C. and maintained at these temperatures. At monthly intervals samples were taken from these cultures and

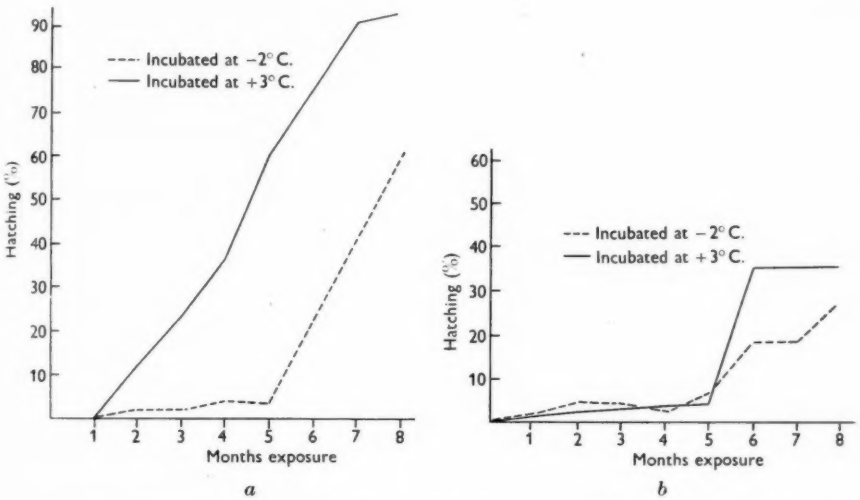

Fig. 4*a*. Percentage hatch of *N. filicollis* infective larvae after incubation at low temperatures.

Fig. 4*b*. Percentage hatch of *N. battus* larvae after incubation at low temperatures.

returned to 21° C., and the percentage hatch after 14 days was recorded, preliminary observations having shown that very little hatching occurred after this time. The viability of unhatched larvae was checked by rupturing the membranes and inducing activity, and 95 % of the larvae were found to remain viable throughout the experimental period. A control culture from the same source was maintained at 21° C., and showed only a 5 % hatch of *N. battus* and 2½ % hatch of *N. filicollis* during the 8 months of observation.

Results

The results are shown graphically in Figs. 4*a*, *b*. Following exposure to low temperature a percentage of *N. battus* larvae hatched on return to 21° C., the percentage hatch increasing steadily with the length of exposure to the low temperature, and reaching 90 % after 7 months. *N. filicollis* larvae showed a similar behaviour, but the percentage hatch remained low until 6 months had elapsed and then rose rapidly to 35 %, and remained at that level. In both instances a higher percentage hatch occurred in the culture maintained at 3° C. than in that maintained at −2° C.

(*b*) *Hatching after exposure to low temperature under natural conditions*

Method and material

In the work described in the previous section eggs were recovered from faeces set out at different periods, and in Series 1–3, from August onwards, a high percentage of fully developed eggs was recovered. These eggs were incubated at 21° C and examined after a month's incubation to determine whether hatching had occurred.

Table 3. *Percentage hatch of fully developed eggs of* Nematodirus battus, *recovered in Series* 1–3, *after incubation at* 21° C.

(Set 1 = faecal pellets; set 2 = liquid faeces.)

Date of recovery	Series 1		Series 2		Series 3		Mean
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2	
14 Aug.	1	2	0	4	0	3	2
19 Sept.	3	0	0	1	0	1	1
14 Oct.	4	0	4	5	1	1	2·5
14 Nov.	45	45	19	28	24	28	31
14 Dec.	77	90	54	62	47	68	66
14 Jan.	47	91	72	93	100	92	82
14 Feb.	96	98	96	94	95	93	95
18 Mar.	96	98	98	100	100	98	98

Results

The results (Table 3) show that fully developed eggs recovered in August, September and October remained dormant and gave only a very low percentage hatch. From November onwards, however, an increasing percentage of these eggs, fully developed but inactive when they were recovered, became active and hatched when they were raised to 21° C. For *N. battus* the percentage hatch rose rapidly from a mean of 2·5 % in October to 66 % in January and 95 % in February, while for *N. filicollis* the percentage hatch rose more slowly, to 55 % in February, and 84 % in March. Thus a proportion of the infective larvae appeared ready to hatch after quite a short exposure to low temperature and presumably they were only prevented from hatching naturally by the fact that maximum winter temperatures were too low to provide a sufficient stimulus.

(c) *The hatching response at different temperatures*

The temperature at which the larvae hatched in the previous experiments, namely 21° C., was considerably higher than would be experienced under natural conditions in the spring. A further experiment was therefore designed to investigate the hatchability of the larvae at different temperatures.

Table 4. *Percentage of fully developed eggs of* Nematodirus filicollis *recovered in Series* 1–3, *after incubation at* 21° C.

(Set 1 = faecal pellets; set 2 = liquid faeces.)

Date of recovery	Series 1		Series 2		Series 3		Mean
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2	
14 Aug.	4	0	0	0	0	0	0·7
19 Sept.	0	0	0	0	0	3	0·5
14 Oct.	1	0	0	0	0	0	0·1
14 Nov.	6	7	18	8	12	1	8·7
14 Dec.	3	40	24	24	36	27	26
14 Jan.	12	11	5	35	13	11	14·5
14 Feb.	53	42	64	60	50	60	55
18 Mar.	84	82	90	94	80	76	84

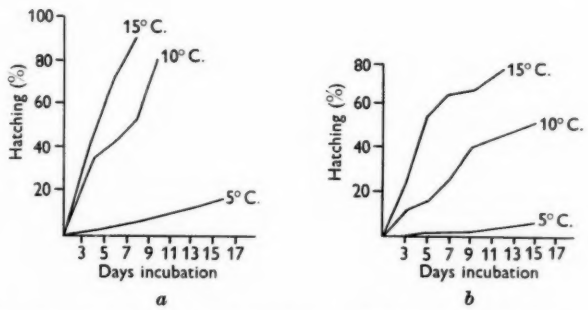

Fig. 5*a*. *N. battus*, rate of hatching at different temperatures.

Fig. 5*b*. *N. filicollis*, rate of hatching at different temperatures.

Material and method

For this purpose fully developed eggs were taken from the material recovered on 18 March from Series 1–3, these eggs having been exposed to a low temperature throughout the winter. Cultures of 50 eggs were incubated in water at 5, 10 and 15° C., four to five cultures of each species being used at each temperature. The cultures were examined at intervals of 2 to 3 days and the percentage of larvae hatching was recorded.

Results

The results are shown graphically in Figs. 5*a*, *b*. At 5° C. (41° F.), *N. battus* eggs hatched slowly and in small numbers. At 10° C. (50° F.), 80% of the larvae hatched in 9 days, while at 15° C. (59° F.), 95% of the larvae hatched in 7 days.

N. filicollis showed similar results, but hatching was slower in all cases, a maximum hatch of 75 % being recorded after 12 days incubation at 15° C. Thus eggs which had overwintered in the third larval stage were stimulated to hatch at temperatures as low as 10° C. (50° F.). It was noticeable that hatching did not occur immediately on exposure to a higher temperature, and the influence of temperature appeared to be to some extent cumulative.

DISCUSSION

The results of Series 1–6 may be considered in two groups, those for Series 1–3, begun in the spring–summer, and those for Series 4–6 begun in the autumn. In the first group development was slow in May, slightly faster in June, and then proceeded relatively rapidly during July–September. Mean temperatures were quite similar throughout May–September, and the higher rate of development appears to be related to the higher rainfall in July–September, averaging 1·26 in. per week as compared with 0·16 in. in May and 0·73 in. in June. Development to the third larval stage was completed in a minimum of 2 months, the majority of the eggs requiring 3 months exposure to reach this stage, even under favourable climatic conditions.

Some development occurred in Series 3–4 during the period 19 September–14 November, mean maximum temperatures during this time being in the range of 49–64° F.; but little development occurred during November–March. Weekly mean maximum temperatures between 10 November and 9 March ranged from 39 to 49° F., except for a single week in December, when 53·9° F. was recorded; and it would appear that for both species development is inhibited under field conditions at temperatures below 50° F. Eggs in all stages of development were extremely resistant to winter conditions.

The striking feature of these results is the fact that, although considerable numbers of fully developed eggs were present in Series 1–2 in August and September, almost none of these eggs hatched, despite favourable conditions of high rainfall and weekly mean maximum temperatures of 56–65° F. The following spring, however, large numbers of hatched larvae were recovered in the period March–June. Temperatures in March were considerably below those recorded the previous September, and hatching could therefore not have occurred as a simple reaction to temperature. The laboratory experiments indicate that the mechanism involved is a sensitizing of the larvae by prolonged exposure to low temperature, resulting in hatching when the temperature is subsequently raised. That the sensitization effect is not merely due to ageing is shown by the fact that fully developed eggs maintained at 21° C. did not hatch throughout a 6-month period of incubation.

The laboratory experiments showed that following exposure to low temperature throughout the winter, hatching occurred in a high proportion of eggs at 10° C. (50° F.) and 15° C. (59° F.) over a period of 9 days, but that at 5° C. (41° F.) very little hatching occurred. This suggests that following sensitization there is a critical temperature at which hatching occurs, and that this temperature is around 50° F. These experiments also showed that several months exposure to low temperature

is required before hatching can be induced, so that under natural conditions few larvae would be capable of hatching until November–December. At this time maximum temperatures are generally below 50° F., and conditions are such that little hatching would occur until the temperature rises in the spring. An analysis of meteorological data showed that in 1957 the weekly mean maximum temperature rose above 50° F. for the first time in the week 9–16 March, reaching 56·6° F. This coincides markedly with the rise in larval counts in Series 1–3, which generally began in samples taken on 18 March, and with the rise in the larval count on the adjacent experimental plot, which also began on 18 March. Weekly mean maximum temperatures are taken because the laboratory experiments indicate that the temperature effect is cumulative, 7–9 days exposure being required for a high percentage hatch.

An analysis of results given previously for *Nematodirus* larval counts on natural pastures (Thomas, 1960*b*) shows a similar trend. These observations were carried out at Nafferton, Northumberland, during 1955–57. In 1955, the pasture larval count began to rise between 22 and 30 April, while the weekly mean maximum temperature rose above 50° F. for the first time in the week ending 9 April. In 1956 the larval count rose sharply between 31 March and 9 April, and the weekly mean maximum temperature rose above 50° F. for the first time in the week ending 3 April, rising to 55° F. In 1957, the larval count rose during the period 12–20 March, while the weekly mean maximum temperature was 49° F. in the week ending 14 March, and 57° F. in the week ending 18 March, again being above 50° F. for the first time that year. Thus in each year the beginning of the seasonal rise in pasture larval count coincided with the onset of weekly mean maximum temperatures of above 50° F. The delay of 2 weeks between the temperature reaching 50° F., and the beginning of the rise in larval count in 1955, is possibly due to the fact that an abnormally low rainfall was recorded in these 2 weeks.

Thus the critical factor involved in the life history of these two species appears to be the necessity for a period of low-temperature exposure before hatching will occur. This mechanism offers a satisfactory explanation for the most puzzling features of the life cycle, namely the marked seasonal incidence of infection and the abnormally long life cycle involving only one generation per year. Eggs are deposited on pasture in the spring and summer of one year and develop to the infective stage, but require exposure to winter temperatures before hatching to infect new hosts the following spring. Small numbers of hatched larvae appear from September onwards, but these are probably partly due to accidental rupture of the membranes, and partly to the fact that a small proportion of larvae are capable of hatching at temperatures below 50° F., and even down to 41° F., as the laboratory experiments show.

The results of grazing worm-free lambs during July–October on a plot contaminated the previous May–June showed that no infection was picked up, although fully developed eggs must have been present on this plot at least during August–September. Thus it would appear that the unhatched eggs do not normally infect the grazing animal, although Gibson (1958) showed that fully developed eggs of *N. battus*, but not those of *N. filicollis*, could be infective under experimental

conditions. The absence of infection under natural grazing conditions is probably due to the fact that the eggs tend to be washed down into the base of the mat and the top-soil, where they are not available to the grazing animal. The lack of success in infecting sheep experimentally with *N. filicollis* eggs may be correlated with the observation reported in this work that fully developed eggs of *N. battus* hatched at 36–38° C., and would therefore probably hatch on ingestion, whereas those of *N. filicollis* did not.

The existence of a period of obligatory dormancy in the life cycle has not been described previously in the family Trichostrongylidae and explains why the life histories of these two species differ so markedly from those of other members of the family. Crofton (1957) developed a theory of succession of species which fitted all species of intestinal nematodes except *Nematodirus*, and to explain this discrepancy it was suggested that some special factor might be required for the development of each generation, apart from the normal temperature and humidity requirements. The present observations confirm this suggestion and indicate the nature of this special factor. This author also stated that, on theoretical grounds, a second generation could occur about September in most years, but that this did not occur in practice. Results recorded above show that a high proportion of eggs reach the third larval stage in August–September and in the absence of an obligatory period of dormancy a second generation would occur at this time.

Both species showed a similar pattern of behaviour, but development of the larval stages of *N. filicollis* was more rapid than that of *N. battus* and the former appeared less affected by dry conditions. The results obtained in the hatching experiments were more consistent and clear-cut in the case of *N. battus*, and the graphs of larval activity were also more regular for *N. battus* than for *N. filicollis*, in which hatching appeared to be somewhat erratic. In view of the marked preponderance of *N. battus* in north-east England, it is possible that conditions in this area are much more suitable for *N. battus* than for *N. filicollis*, which is dominant in southern England, and this may account for the less regular results obtained with the latter species in the present work. Although larval development was more rapid for *N. filicollis* than for *N. battus*, the period of peak larval counts was slightly later for this species than for *N. battus* in Series 1–3. However, no such difference was found in the larval counts on the experimental plot, or in those previously carried out on natural pastures.

In Series 2–3 relatively high numbers of *N. filicollis* larvae were recorded in June, after the count on the experimental plot had fallen to a low level. It seems probable that this was due to a delayed hatching of eggs which had begun development in autumn, but only reached the third larval stage the following spring. This could also explain the small numbers of *N. filicollis* larvae recovered from Series 4–5 in July. Where no development occurred in the autumn (Series 5–6), the eggs developed normally the following summer but remained unhatched, and would apparently remain inactive until the following spring. It is thus possible for some infection to be carried over on pasture for 2 years after the year in which contamination occurred.

It was noted in earlier work (Thomas, 1960*b*) that in 1956 a marked double

peak of larval activity occurred on natural pasture, and at the time no explanation for this could be given. The second peak, however, which occurred in June, shows a marked resemblance to the rise in activity in June recorded in Series 3 of the present work, and it is possible that it may have been due to the same cause, namely the delayed hatching of larvae which only completed development in the spring. The summer of 1955 was extremely dry, monthly rainfall averaging 1·15 in. in June–September, as compared with 3·5 in. in 1954 and 4·0 in. in 1956. It has been shown that larval development is very slow under dry conditions and it is possible that an abnormally high proportion of eggs failed to complete their development in the summer of 1955, and required a further period to complete development in the spring of 1956 before hatching could occur, thus giving rise to the second peak of larval activity.

Lapage (1956) stated that the infective larvae of *Nematodirus* species do not readily hatch unless the temperature is above 24° C., and attributed the seasonal incidence of *Nematodirus* infection in Britain to this fact. However, a consideration of meteorological data shows that temperatures above 24° C. (75° F.) are only reached in midsummer in north-east England, while in 1956 the highest weekly maximum temperature recorded was only 66° F., in late July. Thus, if this explanation were correct, peak larval counts would be expected in July–August. In practice peak larval counts are found to occur in March–May, and this suggestion would therefore appear to be incorrect, the more likely explanation being that given in the present work.

Some support for the explanation given in this work is shown by the findings of Poole (1956), that an exposure to −6·5° C. for 48 hr. during incubation shortened the development time of *N. filicollis* eggs by 24–48 hr. This author suggested that the life cycle may be assisted by a cooling stage. Further support is given by the fact that the hypothesis here given adequately explains the marked seasonal nature of *Nematodirus* infection in lambs recorded by Thomas & Stevens (1956) and Baxter (1957) in Britain, and by Tetley (1934–35) in New Zealand. Spedding, Brown & Wilson (1958) found that *N. filicollis* larval counts on pasture in southern England began to rise in January, but that peak larval counts varied from January to April, confirming the rather erratic hatching behaviour observed in this species in the present work.

Goldberg & Rubin (1956) investigated the survival of *N. filicollis* infection on pasture in Maryland, U.S.A., by grazing susceptible calves on contaminated pasture at intervals after contamination. The results showed that of the eleven species present in the animals used to contaminate the pasture in July and August, *N. filicollis* was the only species which did not appear in the calves grazed in September and November. Calves grazed in December and the following May, however, showed infection with this species. The interval between the December and May grazings was too long to indicate when the peak *N. filicollis* burden would have occurred, but the observations generally agree with the results of the lamb-grazing experiment reported in this work.

In the case of *N. spathiger*, however, a similar experiment carried out by Kates (1950), in Maryland, U.S.A., showed that lambs acquired a *Nematodirus* burden

when grazing within 3 weeks of the date of contamination of the pasture. It was found that the *Nematodirus* burden reached a maximum in lambs grazed 1–2 months after contamination had occurred, and that, although overwinter survival of larvae occurred, only small numbers of adult worms were recovered from lambs grazing the following spring. This is in marked contrast to the observations on *N. filicollis* and *N. battus*, and indicates that the eggs of *N. spathiger* must develop to the infective stage in 2–3 weeks on pasture and then hatch immediately. This confirms laboratory observations (Thomas, 1960*a*); Kates & Turner, 1955) that *N. spathiger* eggs developed more rapidly than those of *N. battus* and *N. filicollis*, and that the eggs hatched readily without undergoing any period of quiescence. Thus the phenomenon of delayed hatching does not appear to apply to *N. spathiger*, which is the common species in North America, and this would account for the difference in etiology of *Nematodirus* disease in sheep in America and Britain.

Both Kates (1950) and Goldberg & Rubin (1956) considered that the infective larvae of *Nematodirus* species could remain inactive within the egg membranes under dry conditions, and only hatched under favourable conditions of temperature and moisture. This would appear to be true for *N. spathiger*, and probably for the related species *N. helvetianus* in cattle, but the hatching requirements of *N. battus* and *N. filicollis* are much more stringent and a long period of dormancy is an essential part of the life history. It is possible that a gradation occurs between *N. spathiger*, in which hatching normally occurs rapidly, *N. filicollis* in which some delay always occurs but hatching is erratic in late winter–spring, and *N. battus* in which hatching is constantly and uniformly delayed until spring.

In some respects this type of behaviour resembles the phenomenon of diapause in insects, being a phase in the life history enabling the organism to withstand unfavourable conditions, and synchronizing the life cycle with that of the host. In this case the dormancy period enables the infective larva to overwinter within the protection of the egg membranes, and hatching occurs in the spring, coinciding with the presence of large numbers of highly susceptible lambs. In addition, the activation of the organism by prolonged exposure to low temperature is a typical feature of the diapausing insect. However, diapause is a complex phenomenon during which important physiological changes occur, and, if the insect is disturbed during diapause, it usually fails to complete development. The infective larvae of *Nematodirus* species, however, appear to undergo only a resting stage, and if this is interrupted or prevented by artificially hatching the larvae, no abnormality results. Successful infections can also be established by using larvae which have not undergone a resting stage. Thus this period of dormancy is probably best described as 'quiescence' and is not to be classed a true diapause.

SUMMARY

1. The availability of *Nematodirus* infection to sheep was followed by grazing successive pairs of worm-free lambs on a contaminated plot. It was shown that lambs did not become infected during the summer following pasture contamination in the spring, but became infected when they were grazed on the plot the following spring.

2. The development and hatching of the eggs and larval stages of *N. battus* and *N. filicollis* were followed by the periodical examination of infected faeces set out during May–November. The results showed that development to the infective larval stage occurred during the summer, taking normally about 3 months, but that the larvae then remained dormant within the egg membranes until the following spring.

3. Laboratory studies confirmed the hypothesis that the hatching of the infective larvae was a response to a rise in temperature in the spring, following sensitization by exposure to low temperature during the winter. A rise in temperature to 50° F. was necessary before a high percentage hatch occurred.

4. The epidemiology of *Nematodirus* infection is discussed in relation to these observations.

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With 1 *plate and* 4 *text-figures*
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The attachment of the monogenean *Discocotyle sagittata* Leuckart to the gills of *Salmo trutta* L.

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(*Received* 13 *March* 1959)

Recent studies of the ways in which diclidophoroidean trematodes attach themselves to the gills of their hosts have shown that there are at least two kinds of clamping mechanism: one consists of an extrinsic muscle–tendon–fair-lead–hinged-jaws system (e.g. *Plectanocotyle gurnardi* and *Kuhnia scombri*) and the other an extrinsic muscle acting upon a diaphragm to produce a suction pressure which closes a pair of hinged jaws (e.g. Diclidophoridae) (Llewellyn, 1956*a*, 1957*a*, 1958). Both these mechanisms differ from those proposed by Scheuring (1938) and by Sproston (1946) in their explanations of the mode of action of the clamps of *Discocotyle sagittata* (Leuckart, 1842) Diesing, 1850, during the attachment of the parasite to the gills of *Salmo trutta* L., and so a re-investigation of the attachment of *Discocotyle* to its host has been undertaken.

At the same time another problem of the attachment of *Discocotyle* to trout gills has been investigated. In describing the comparative morphology of some gill trematodes (Llewellyn, 1956*b*, 1957*b*) an explanation was offered for the frequent occurrence of asymmetry of the body, but the apparently bilaterally symmetrical *D. sagittata* remained an enigma, and so this anomalous condition has been examined anew.

Fresh fishes for examination were obtained from Lakes Coniston and Windermere, and from rivers in Montgomeryshire and Cardiganshire. Observations on freshly caught living material were made at the Windermere laboratory of the Freshwater Biological Association, and at Penybontfawr (Montgomeryshire), the shortest interval between the capture of the host and the examination of the parasites being 10 min. In addition, heads and gills from trout which had been killed by immediate immersion in Bouin's fluid were supplied by the Freshwater Fisheries Laboratory, Pitlochry (Perthshire). These preserved specimens provided a supply of histologically fixed parasites attached to the host as in life.

Detailed records were kept of the precise sites of attachment of the parasites to their hosts (see Llewellyn, 1956*b*, p. 114). The histological techniques were those previously employed for *Plectanocotyle*, *Kuhnia* and *Diclidophora*.

MICROHABITAT AND ADHESIVE ATTITUDE

In all, 156 adult parasites were collected from sixty fresh host specimens, and were found to be distributed as shown in Table 1. Records from the preserved fishes from Pitlochry have not been included: some of the parasites had become detached before the examination of the hosts, and the observed lower rate of infestation (25 % of 75 trout examined) and the smaller mean number of parasites per infested fish (1·5) could well have reflected the collecting procedure rather than the natural extent of parasitism.

Table 1. *Distribution of* Discocotyle sagittata *among the different gill arches of* Salmo trutta

Locality (see text, p. 51)	No. of fishes examined	No. of fishes infested	Percentage infestation	Total no. of parasites per gill arch				Mean no. of parasites per infested fish
				1	2	3	4	
Wales	24	12	50	14	18	12	7	4·25
Lake District	36	24	67	44	37	14	10	4·375
Totals	60	36	60	58	55	26	17	4·33

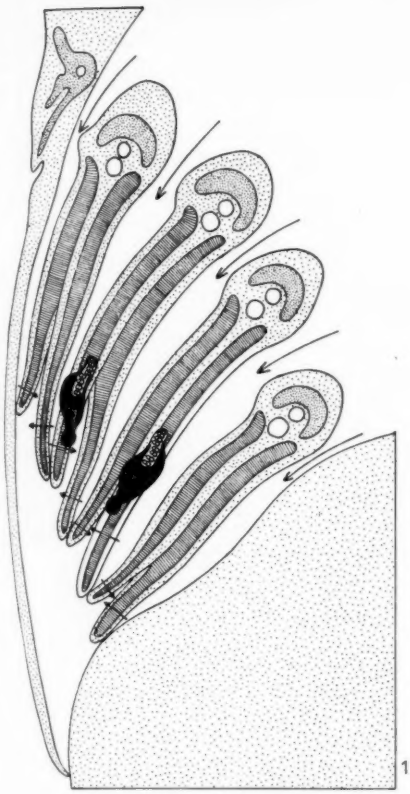
Table 1 shows that about one-half to two-thirds of the fishes in the areas sampled in the Lake District and in Wales each carried about four or five specimens of *D. sagittata*, the parasite exhibiting a preference for the first and second gill arches.

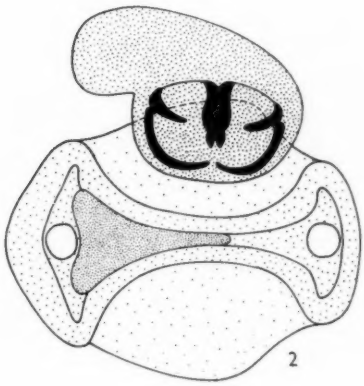
Of the ninety-six parasites whose precise sites of attachment were recorded in detail, ninety-two were attached to secondary gill lamellae in the vicinity of the distal border of the interbranchial septum (Text-fig. 1, Pl. 1, fig. 2), and the remaining four were attached more proximally.

The parasites adhered to their hosts, as described previously (Llewellyn, 1956*b*, pp. 117–18), with the posterior end nearer to the gill arch, i.e. relatively up-stream in the gill-ventilating current. Nearly all of them were attached to one surface only of a primary lamella; the exceptions will be described later (p. 55).

A detailed examination of the attached specimens of *D. sagittata* revealed that there was a characteristic adhesive attitude in which the median longitudinal axis of the parasite was not parallel to the long axis of the primary lamella, but was inclined to it in such a manner that the anterior end of the body was always directed towards the space between the two hemibranchs of the particular gill arch to which the parasite was attached (Text-fig. 1). Thus, the direction of inclination was either to the parasite's left or to its right, dependent upon whether the trematode was attached to an inner or to an outer hemibranch, and whether to the dorsal or to the ventral surface of a primary lamella. The precise sites of attachment of a sample of fifty parasites, together with their inclinations to left or right, are given in Table 2.

From Table 2 it may be seen that there appeared to be some preference for attachment to an inner hemibranch (31 specimens) rather than to an outer (19),





Text-fig. 1. Horizontal section through one side of branchial region of *Salmo trutta* to show typical sites of attachment of *Discocotyle sagittata* and paths of gill ventilating currents. (Planes of primary lamellae modified for purpose of diagram.)

Text-fig. 2. The relative sizes of a clamp of *Discocotyle sagittata* and the secondary gill lamellae of *Salmo trutta*.

Table 2. *Direction of the inclination of the longitudinal axis of the body and the precise sites of attachment of a random sample of fifty specimens of* Discocotyle sagittata *to the gills of* Salmo trutta

(For explanation of the terms 'right' and 'left' see Text-fig. 1 and text, p. 52.)

		Dorsal side of primary lamella	Ventral side of primary lamella
Right gill chamber	Outer hemibranch	Right (3)	Left (4)
	Inner hemibranch	Left (8)	Right (6)
Left gill chamber	Outer hemibranch	Left (7)	Right (5)
	Inner hemibranch	Right (8)	Left (9)

but that there was no preference for attachment to one surface of a primary lamella rather than to the other (dorsal surface 26, ventral 24), and no marked tendency for the body to be inclined more towards one side than to the other (28 to the parasite's left, 22 to the right).

When preserved parasites were removed carefully from the gill, the inclination of the longitudinal axis of the parasite to that of the primary lamella was seen to be accompanied by a markedly asymmetrical condition of the posterior adhesive apparatus: the four clamps of one side of the body were all situated anterior to the four clamps of the other side (Pl. 1, fig. 1). The characteristic distribution of 'footprints' left on the host by such an adhesive apparatus is shown in Pl. 1, fig. 2. The more posterior set of clamps is invariably on the same side of the body towards which the longitudinal axis is inclined, and the corresponding set of 'footprints' always nearer to the space between the two hemibranchs belonging to the same gill arch.

A comparison of the sizes of the clamps of the parasite and of the secondary lamellae of the host (Text-fig. 2) shows that it is not possible for a secondary lamella to accommodate two clamps side by side; this applies to any one of the whole series of secondary lamellae on all the primary lamellae of all the trout up to 30 cm. in length which we have examined. The inability of the parasite to attach both members of a pair of clamps to the same secondary lamella is, however, possibly a consequence of an alignment inclined to the primary gill lamella rather than a cause of it. If the asymmetry of the posterior adhesive apparatus were due only to the size-relationship between parasite and host, the resulting inclination of the body might be equally to the left or right; but since the direction of inclination is always so as to bring the anterior end of the parasite nearer to the inter-hemibranchial space, some other factor must operate, and this factor is almost certainly the path of the ventilating current over the gills.

In an earlier paper (Llewellyn, 1957*b*), brief reference was made to the relationship of the gill ventilating current, as described in the comparatively little-known but very important work of Woskoboinikoff, to the body symmetry of monogenean gill parasites. Woskoboinikoff's interpretation of the gill ventilating mechanism of teleosts has been very largely substantiated by some recent ingenious experiments by Hughes & Shelton (1958), who, in the course of describing their own work, have also given a concise review of the subject. Briefly, the gill ventilating mechanism consists of a median buccal force pump and a pair of lateral opercular suction pumps, the two pumping systems being separated from each other by a continuous sieve formed by the interdigitation of the primary lamellae of adjacent hemibranchs. In this system, excepting during occasional 'coughing', all water passing from the mouth cavity into the opercular cavities is obliged to pass through the meshes of the sieve, and in particular, is caused to flow in the channels between adjacent *secondary* lamellae, i.e. at right angles to the long axes of the primary lamellae. Moreover, the water continues to flow from the buccal to the opercular cavities throughout about nine-tenths of the duration of each cycle of ventilating activities.

Thus, any parasites attached to a gill at a position distal to the interbranchial septum will be subjected to an almost continuous flow of water passing inwards into the space between the two hemibranchs belonging to the same gill arch, as illustrated in Text-fig. 1. *D. sagittata* reduces its resistance to this unilaterally incident current by taking up an alignment inclined to the long axis of the primary lamella, the asymmetrical disposition of the adhesive organs helping to bring this about. The habit of attaching only one clamp to each secondary lamella (or group of two or three secondary lamellae) could have permitted the (phylogenetic) development of increased efficiency of the clamping apparatus to take the form of increased size of clamps rather than, say, an increase in the number of clamps as appears to have taken place in the Gastrocotylidae and Microcotylidae.

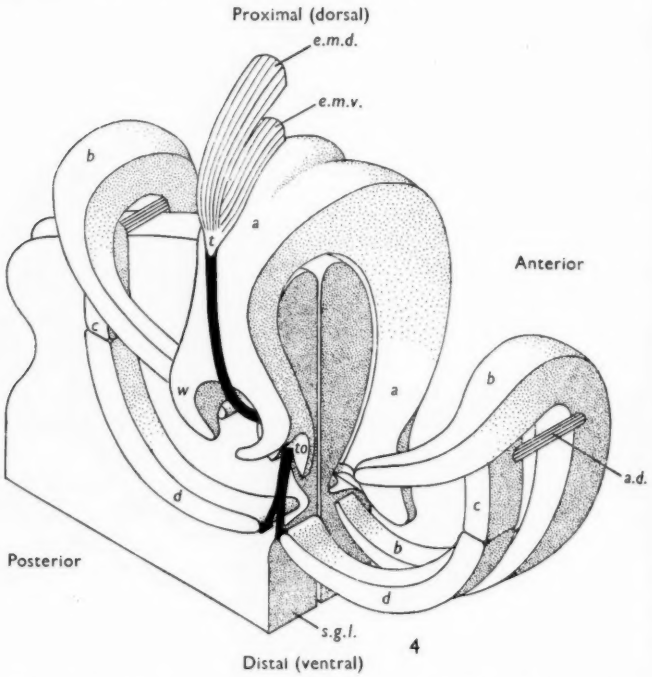
Observations on fresh living specimens which had been forcibly detached from the gill tissue have shown that the parasite is able to re-attach itself, and in so doing to be capable of assuming (in the absence of a gill ventilating current) an alignment different from that previously adopted. Moribund specimens which had, without interference, become free from the host tissue were usually found to be bilaterally symmetrical, as were the dead specimens collected (see photograph in Llewellyn, 1956*b*, pl. I, fig. 3). It is noteworthy that in all previous references to *D. sagittata*, e.g. Leuckart (1842), Shaffer (1916), Scheuring (1938), and Llewellyn (1956*b*), the posterior adhesive apparatus has been illustrated as being bilaterally symmetrical. The inference is that the specimens used for these descriptions were moribund or dead upon collection.

It may be concluded, therefore, that *Discocotyle* is facultatively asymmetrical either to its left or its right, and not obligatorily and permanently asymmetrical to one side only as has been illustrated previously for *Anthocotyle*, *Gastrocotyle*, *Pseudaxine* and *Axine* (Llewellyn, 1956*b*). Facultative asymmetry to one side or to the other would be less specialized than obligatory unilateral asymmetry, and could well represent the survival in *Discocotyle* of a primitive character.

Two conditions of symmetry not conforming with the above general situation were encountered. The first was when two parasites, each attached to the proximal region of a primary lamella, were found to be bilaterally symmetrical. Here the shelter afforded by the interbranchial septum made asymmetrical alignment to a unilateral current unnecessary, as in *Kuhnia scombri*. The problem of sufficient space to accommodate two clamps at the same level had been overcome by the clamps of one side becoming attached to secondary lamellae at the corresponding level of a different, but adjacent, primary lamella of the same hemibranch. The second exception was when two parasites were found to be asymmetrical in the reverse direction from the typical state described above and recorded in Table 2. These parasites were again found in the shelter of the interbranchial septum, but this time were attached to secondary lamellae of one surface of a single primary lamella. It has been shown above that the secondary lamellae are too narrow to accommodate two clamps side by side and so the parasites were obliged to attach each of the two members of a pair of clamps to different secondary lamellae, i.e. lamellae at different distances from the gill arch. The currents in the shelter of the interbranchial septum are probably too weak to influence the orientation of the

parasite, and so presumably by chance these two parasites had taken up an alignment different from that of typical exposed parasites attached more distally along the primary lamella.

Repeated observations on perfectly fresh specimens failed to support the statement made by Scheuring (1938) to the effect that *Discocotyle* led by no means a fixed life and moved about very much like a leech. Not one *Discocotyle* was seen voluntarily to detach itself and then to become re-attached. However, as mentioned previously, parasites which were forcibly detached were observed to re-attach themselves, though usually by the clamps of one side only.



Text-fig. 3. One of the hooks (= persistent larval posterior hook) of an adult *Discocotyle sagittata*.

Text-fig. 4. Stereogram of a clamp of *Discocotyle sagittata* grasping secondary gill lamellae of *Salmo trutta*. (The lamellae are represented as being cut vertically in the sagittal plane of the clamp.) *a.d.*, intrinsic adductor muscle; *e.m.d.*, extrinsic muscle running to dorsal region of body proper; *e.m.v.*, extrinsic muscle running to ventral region of body proper; *s.g.l.*, secondary gill lamella; *t*, tendon; *a*, *b*, *c*, *d*, *to*. (= 'tongue'), *w*, sclerites or parts of sclerites as described in text, p. 57.

ADHESIVE MECHANISM

Discocotyle sagittata attaches itself to the secondary lamellae of the gills of its host mainly by means of four pairs of posteriorly situated clamps (Pl. 1, figs. 1–4), but there is in addition a pair of relatively small hooks. These hooks (Text-fig. 3) are about 68μ long and are similar in shape and size to the posterior hooks of the

newly hatched larva; similar hooks have been observed in post-larval but immature specimens of *Discocotyle*. There can be no doubt but that these hooks persist with little if any alteration throughout life, and make some small contribution to the attachment of the adult parasite to the host. It is noteworthy that these hooks bear a greater resemblance to the posterior hooks of the larvae of *Anthocotyle* and of the Microcotylidae (Llewellyn, 1957*c*) than they do to other diclidophoroideans.

In newly hatched oncomiracidia there may be seen sometimes a pair of slender hooks anterior to and slightly lateral to the hooks described above, but these 'postero-lateral' hooks (see Llewellyn, 1957*c*, p. 247) do not appear to persist in the adult.

Each clamp is composed of two jaws, an anterior fixed jaw and a posterior hinged jaw, supported by a system of sclerites as indicated in the stereogram (Text-fig. 4), and is generally similar to the clamp of *Plectanocotyle* (Llewellyn, 1956*a*, text-fig. 1).

The median sclerite (*a*) is a single structure which at its anterior, ventral end is T-shaped, and at its posterior end bears a 'fair-lead'. In this region the postero-lateral parts of the walls of sclerite *a* are continued distally as 'wings' (*w*) whose tips are curved towards each other but do not meet. The basal (anterior) part of sclerite *a* in the fair-lead region is continued distally beyond and beneath (i.e. anterior to and nearer the cavity of the clamp) the in-curved wings as a tongue-like extension (*to.*) with a median longitudinal channel in its posterior surface. In its proximal region the channel is roofed-over by a thin bridge of sclerotized material, and through the canal thus formed passes a tendon (*t*). Overlying the thin sclerotized bridge, i.e. in the region between the wings and the channelled tongue, is a mass of fibrous tissue continuous with that composing the walls of the clamp. Distally, beyond the end of the channel, the tongue-like extension of sclerite *a* is forked, the tips of both prongs being inclined slightly posteriorly, i.e. away from the cavity of the clamp. The tendon which passes along the channel connects the extrinsic muscles of the clamp with the peripheral sclerites *d* of the movable jaw. The tendon bifurcates distally, one branch going to the postero-ventral edge of the distal (median) regions of each of the two peripheral sclerites *d*.

The peripheral sclerites of the fixed jaw (*b*) are separated from each other by a narrow gap anteriorly, and posteriorly approach the channelled-tongue of the fair-lead but do not meet it. The parts of these sclerites anterior to the hinge joint are hollow, but posterior to it they are solid and more slender.

The peripheral region of the posterior jaw is supported by a pair of sclerites (*d*) free from each other distally and jointed along their lengths so as to form a shorter proximal distinct, but fused, sclerite *c*. A few clamps were observed in which *c* and *d* did not adjoin each other directly but were separated from each other by a very small sclerite.

In the hinge region of the clamp there is on each side a small intrinsic adductor muscle (*a.d.*) connecting sclerites *b* and *c*.

Closure of the clamp is effected by the contraction of the two sets of muscles, the intrinsic muscles (*a.d.*) and the much more powerful extrinsic muscles (*e.m.d.*, *e.m.v.*). Of the latter, there are two main components, one bundle from the ventral

region of the body of the parasite (*e.m.v.*), and the other from the dorsal (*e.m.d.*). Contraction of the extrinsic muscles would pull the tendon through the fair-lead and so swing the movable jaw anteriorly about its hinges. Other muscles are associated with the clamps; some connect the 'wings' of the fair-lead region with the hinge-joint regions of the anterior (fixed) jaw of the succeeding clamp and are probably concerned with the orientation of the clamp; others pass from the distal median region of the movable jaw (*d, d*) to the dorsal body wall of the parasite and could serve to open the clamp.

There is then a very close correspondence between the sclerites of *Discocotyle* and those of *Plectanocotyle*; the main differences are: (1) in *Discocotyle* the peripheral sclerites of the posterior jaw (*d, d*) are free from each other distally, but in *Plectanocotyle* there is a single lamellate sclerite *d* of relatively greater anterior-posterior dimensions; (2) there is a greater development of sclerotized tissue in association with the fair-lead region of *Plectanocotyle*. Both these differences would appear to be in the direction of a mechanically more efficient clamp in *Plectanocotyle*; while *Discocotyle* is most certainly quite successful in grasping firmly the gill tissue of its host, the apparently more efficient mechanism in *Plectanocotyle* suggests that this parasite might achieve a corresponding result with less effort.

It is concluded that the clamping mechanism of *Discocotyle sagittata* consists of an extrinsic muscle–tendon–fair-lead–hinged-jaws system, and that Scheuring's (1938) and Sproston's (1946) interpretations of the mechanism as a spring system are wrong.

SUMMARY

The monogenetic trematode *Discocotyle sagittata* attaches itself to the gills of *Salmo trutta* with its long axis inclined to the long axis of the primary lamella to which it is attached, the direction of inclination being determined by the incidence of the gill-ventilating current of the host. The inclination is to some extent brought about by an asymmetrical disposition of the posterior adhesive organs, but *Discocotyle* appears to retain a primitive unspecialized character in that the asymmetrical disposition is facultative and not obligatory and permanent as in *Axine*.

The adhesive apparatus consists of four pairs of clamps and one pair of hooks. The hooks appear to be the persistent posterior hooks of the larva.

Each clamp is operated by an extrinsic muscle–tendon–fair-lead–hinged-jaws system generally similar but possibly less efficient than the corresponding system in *Plectanocotyle gurnardi*. Scheuring's and Sproston's interpretations of the clamp-closing mechanism of *Discocotyle* as a spring system are wrong.

We would like to express our thanks to the Director of the Freshwater Biological Association, Ambleside, and to the Director and to Mr K. A. Pyefinch of the Freshwater Fisheries Laboratory, Pitlochry, for laboratory facilities and for supplying some of the material used in this work. One of us (I.L.O.) was in receipt of a Department of Scientific and Industrial Research grant.

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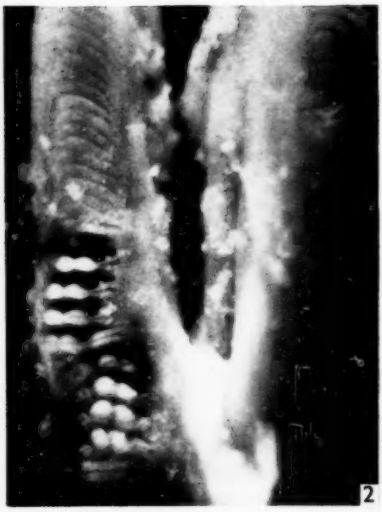
EXPLANATION OF PLATE 1

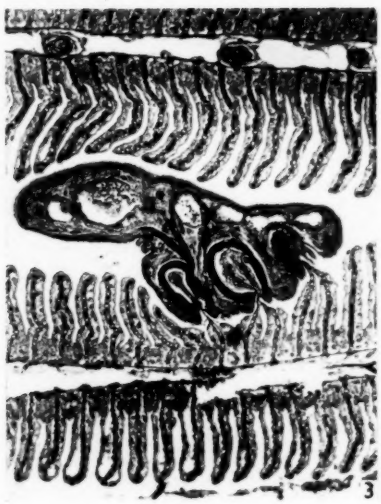
Fig. 1. *Discocotyle sagittata* in ventral view, showing asymmetrical disposition of posterior adhesive organs; specimen fixed in its characteristic adhesive attitude.

Fig. 2. Interbranchial septum region of gill of *Salmo trutta* with 'footprints' of *D. sagittata* (same specimen as shown in fig. 1, and to same scale).

Fig. 3. Parasagittal section of *D. sagittata* attached to secondary gill lamellae of *S. trutta*.

Fig. 4. Sagittal (slightly oblique) section of clamp of *D. sagittata* showing extrinsic muscles, tendon, and fair-lead. (Anterior-most of the clamps shown in fig. 3.)





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With 1 *plate and* 4 *text-figures*
Printed in Great Britain

Albertia vermicularis (Rotifera) parasitic in the earthworm *Allolobophora caliginosa*

By B. REES

Zoology Department, University College, Cardiff

(*Received* 19 *March* 1959)

INTRODUCTION

Although the majority of rotifers are free-living, there are many records of rotifers parasitizing both plants and animals (Thompson, 1892; Budde, 1925; Remane, 1929). The most marked development of this parasitic habit is found in the genera *Balatro*, *Drilophaga*, *Zelinkiella* and *Albertia*. In these forms, with the exception of the genus *Zelinkiella*, attachment to the host is performed by the mastax. *Albertia vermicularis* Dujardin (1838) has been recorded as a parasite of the intestine and body cavity of earthworms. This author also found specimens in slugs, but he did not indicate the host species involved in either case. This original description was rather incomplete, and it is the object of this paper to expand it by a description of specimens from *Allolobophora caliginosa*.

MATERIAL AND METHODS

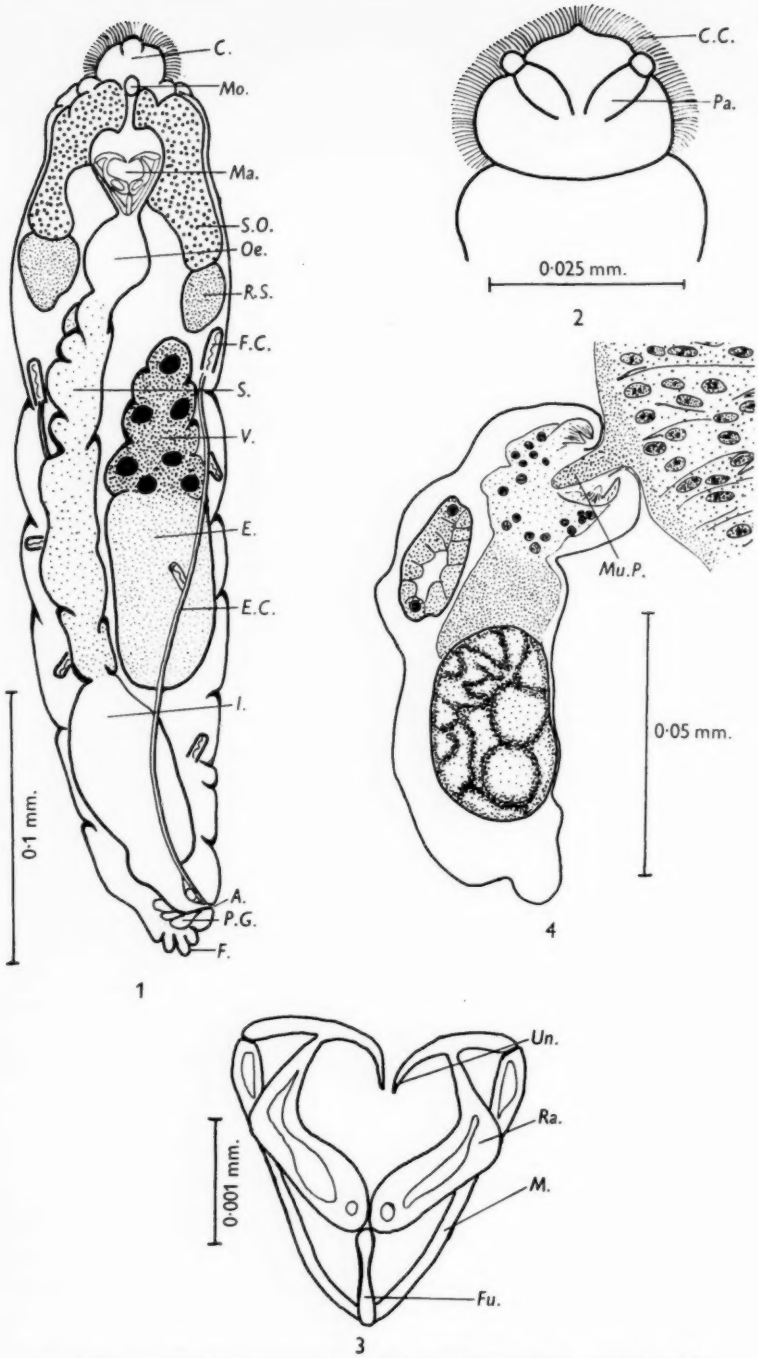
The specimens of *Albertia vermicularis* were found during a survey of the parasitic fauna of certain earthworms. They occurred in only one out of ninety specimens of *Allolobophora caliginosa* obtained from the flower beds at Roath Park, Cardiff.

Each earthworm was dissected alive, under earthworm saline (Pantin, 1946). The intestine was then opened by a median dorsal incision along its length and examined for parasites under a binocular microscope. Areas of infected tissue were removed, fixed in Bouin's fluid, sectioned and stained using standard methods, in order to inspect the relationship between the parasite and the host tissue.

Living specimens, removed from the gut and mounted in saline, were photographed using Electronic Flash (Erasmus, 1958).

MORPHOLOGY

The parasite was found attached to the intestinal epithelium of the host approximately one-third of the length of the worm in front of the anus. Approximately twenty-five specimens were found distributed over about 1·5 in. of the interior of the intestine. They were firmly attached by their anterior ends and it was difficult to detach them without breaking. When detached from the intestinal mucosa of the host, the parasite is able to swim by means of its coronary cilia. The parasite varied in size between $180 \times 35\mu$ and $310 \times 40\mu$; Dujardin's specimens were between 300 and 500μ long. *Albertia vermicularis* is elongated in form and is



Text-fig. 1. *Albertia vermicularis* showing structure from ventral surface.
Text-fig. 2. Dorsal surface of corona, note palps.
Text-fig. 3. Detailed structure of mastax.
Text-fig. 4. Diagram showing host-parasite relationship.
For key see p. 65.

S-shaped in profile. There is no definite segmentation of the body. Anteriorly the corona appears as a hemispherical, simple, unlobed ciliary field (Text-fig. 1; Pl. 1, fig. 1), which is capable of being retracted (Pl. 1, fig. 2). The cilia which are borne on the corona are uniform in distribution and length and were approximately 6μ long in the animals examined. Dorsally, the corona bears two large palps (Text-fig. 2) which are unciliated.

The mouth is situated ventrally on the posterior border of the coronary field and is followed almost immediately by the pear-shaped mastax which is of the virgate type. The mastax in the detached animals is constantly being pushed backwards and forwards between its normal, more posterior, position and the mouth. During these movements the unci are observed to move apart while the mastax is in the region of the mouth (Pl. 1, fig. 2), and to close together when the mastax is completely retracted (Pl. 1, fig. 1). The trophi which are well differentiated and whose detailed form and structure is shown in (Text-fig. 3) are of the same general shape as those of *Balatro anguiformis* Issel (1904). Other prominent structures in the anterior region of the body are the large subcerebral organs and the retrocerebral sacs. These correspond in shape and position to the structures shown in Dujardin's text-figs. 1 and 2, and referred to by that author as anterior and posterior gastric glands respectively. I have found no connexion between these organs and the gut, nor have I been able to locate any organ which corresponds with a gastric gland.

The mastax is connected to the stomach by a short oesophagus which may or may not be dilated. The stomach extends over most of the remaining portion of the body and joins the intestine posteriorly; the intestine may or may not be dilated. The alimentary tract ends in the subterminal anus and connected with it in the posterior region there are four small rounded structures—the pedal glands.

Distributed at regular intervals on either side of the posterior two-thirds of the body are four pairs of long tubular flame cells. Each flame cell is approximately 12μ long and contains a single central flagellum of the same length. The three pairs of anterior flame cells are directly connected with a long excretory canal. The connexion between the posterior pair of flame cells and the main excretory duct has not been observed. Posteriorly the excretory canal connects up with the intestine in the region of the anus. This excretory system, consisting of four pairs of flame cells, corresponds with the description given by Dujardin, i.e., his 'branchies intérieures'.

The germovitellarium is a large structure, the vitellarium containing five to eight large cells. The animal is viviparous, and large specimens often contain an embryo which may half fill the body of the parent individual (Pl. 1, fig. 3). These embryos are often well developed, and details of their internal anatomy may be observed through the body wall of the parent.

Posteriorly the body of the animal tapers towards a blunt point which bears three small lobes, all that remains of the foot which is so typical of various orders of free-living rotifers.

HOST-PARASITE RELATIONSHIP

The position of the specimens in the gut would indicate that this is the normal habitat for this organism.

Dujardin did not mention the way in which *Albertia vermicularis* attaches itself to the host. Sectioned material has revealed however that there is an intimate connexion between the intestinal mucosa of the host and the parasite (Text-fig. 4; Pl. 1, fig. 4). The attachment consists of a small papilla of the intestinal mucosa which has been grasped and held by the trophi within the mastax. It seems from observations on the living animal that it must be the unci which are the trophic parts concerned with the holding, for it is these which are opened and closed during the movements of the mastax in the living animal. Details of the trophi cannot be distinguished in the sectioned material. This method of attachment is similar to that described by de Beauchamp (1904, fig. C) for *Drilophaga delagei* which is ectoparasitic on *Herpobdella octoculata*, in which case the ectoderm of the host is grasped in the form of a small papilla by the trophi of the mastax.

DISCUSSION

The majority of the previously described associations between rotifers and other animals show that the rotifers concerned are either epizoites or ectoparasites on the external surfaces of the host animal. Thus *Proales daphnicola* lives as an epizoite on the valves of *Daphnia* and other Cladocera (Thompson, 1892). The attachment to the host is more intimate in *Balatro anguiformis* Issel (1904) and *Drilophaga delagei* de Beauchamp (1904) which occur on the external surfaces of *Henlea ventriculosa* and *Herpobdella octoculata* respectively. Both these rotifers attach themselves to the host by means of the mastax. This ectoparasitic condition is taken a stage further in *Zelinkiella synaptae* Zelinka (1888), which lives in pits on the skin of sea cucumbers. In this last species the foot lacks toes and is modified to form an adhesive organ.

The present description supports the observations of Dujardin; and the intimate nature of the association between *Albertia vermicularis* and the host tissues indicates that this is a case of a truly endoparasitic rotifer.

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KEY TO LETTERING OF TEXT-FIGURES

A.	anus	*Mo.*	mouth
C.	corona	*Mu.P.*	mucosa papilla
C.C.	coronal cilia	*Oe.*	oesophagus
E.	embryo	*Pa.*	palp
E.C.	excretory canal	*P.G.*	pedal gland
F.	foot	*Ra.*	ramus
F.C.	flame cell	*R.S.*	retrocerebral sac
Fu.	fulcrum	*S.*	stomach
I.	intestine	*S.O.*	subcerebral organ
M.	manubrium	*Un.*	uncus
Ma.	mastax	*V.*	vitellarium

EXPLANATION OF PLATE 1

Fig. 1. Parasite with corona everted, unci closed. × 280.

Fig. 2. Parasite with corona retracted, unci open. × 650.

Fig. 3. Individual containing embryo, note the mastax of the embryo in the posterior region of the body. × 515.

Fig. 4. Photograph of a section, showing the method of attachment of the parasite to the intestinal mucosa of host; note the mucosal papilla being grasped by the mastax of the rotifer. × 1000.

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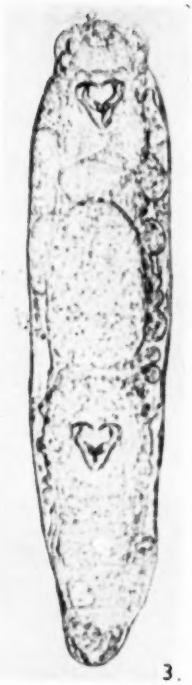

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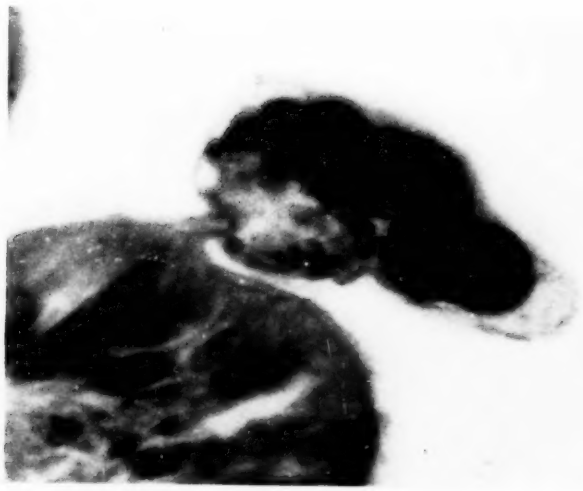

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Studies with *Eperythrozoon parvum* Splitter, 1950

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*School of Veterinary Medicine, University of Cambridge**

(*Received* 6 *April* 1959)

CONTENTS

PAGE
I. Introduction 67
II. Materials and Methods 67
III. Results 68
(1) Staining, morphology, location and size 68
(2) Effect of increased and reduced temperatures 70
(3) Modes of transmission 71
(4) Course of infection 71
(5) Production of anaemia in splenectomized pigs 72
(6) Susceptibility to therapeutic agents 72
IV. Discussion 77
V. Summary 79
References 80

I. INTRODUCTION

Splitter (1950*a*) recorded two new species of eperythrozoa in the blood of American pigs. *Eperythrozoon suis* was responsible for a disease known as ictero-anaemia of swine (Splitter & Williamson, 1950). This organism was closely investigated, but *E. parvum*, which was relatively non-pathogenic, was described only briefly in subsequent papers (Splitter, 1950*b*, 1952, 1953). *E. parvum* has also been recorded in South Africa (Jansen, 1952). Following the detection of *E. parvum* in British pigs (Jennings & Seamer, 1956) the organism was studied in some detail. The principal findings in this investigation are recorded here.

II. MATERIALS AND METHODS

The strain of *E. parvum* used in these experiments was obtained from two experimental litter-mate pigs at the School of Veterinary Medicine, Cambridge.

Most of the pigs used for the experiments were obtained from a self-contained section of the normal breeding herd at the School of Veterinary Medicine. The pigs were reared on an early-weaning system, usually from the first week of life. Pig lice were controlled with benzene hexachloride, and flies were reduced to a minimum with a commercial fly spray. The pigs were splenectomized shortly after weaning. After splenectomy, blood smears were taken from each pig on three alternate days in each week (subsequently referred to as a 'thrice weekly bleeding schedule'). If no eperythrozoa were detected in any smears taken within the 3 weeks

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immediately following splenectomy, the pigs were considered to be free of natural infection with *E. parvum* and were then used for experiment. It was not practicable to examine every pig for the full 3 weeks. When this occurred, approximately half of the litter was examined for 3 weeks, while the remaining animals were inoculated after shorter intervals. Small litters were always examined for the full period of 3 weeks.

Blood for transmission purposes was obtained from the anterior vena cava, using the method of Carle & Dewhirst (1942). Heparin was used as an anti-coagulant in concentrations of 10–20 i.u./ml. of blood. Inocula of 0·5–2 ml. were given intravenously as a routine. For haematological examinations blood was obtained from the tail. Blood counts were made with improved Neubauer haematocytometer slides, haemoglobin was estimated as oxy-haemoglobin in a Unicam spectrophotometer at 542 mμ, and packed red-cell volumes were determined in capillary tubes.

Considerable care was taken to avoid confusing eperythrozoa with artifacts in blood smears. Blood was obtained from the auricular veins after the ears had been cleansed with dry swabs. The needles used for bleeding were sterilized after use on each pig. Blood smears were prepared on clean, grease-free slides. They were fixed in methyl alcohol. The smears were inverted upon curved glass staining trays and stained for 30 min. with Giemsa's stain diluted 1 in 16 with phosphate buffer pH 7·2. After staining they were briefly rinsed in phosphate buffer and dried over an electric lamp. A minimum of forty fields was examined at a magnification of $\times 900$. The results were recorded as uninfected ($-$), lightly infected ($+$), moderately ($++$) or heavily infected ($+++$).

The effect of heat upon *E. parvum in vitro*, and of therapeutic agents *in vivo* was measured by estimating the percentages of lightly infected erythrocytes (one to five eperythrozoa), heavily infected erythrocytes (six or more eperythrozoa) and all infected erythrocytes (sum of those lightly and heavily infected) in blood smears obtained before and after treatment. Two counts of 100 erythrocytes were made on each blood smear, using a standard method, and mean percentages of each value were obtained from the two counts.

III. RESULTS

Unless otherwise stated, all results were obtained from splenectomized pigs.

(1) *Staining, morphology, location and size*

E. parvum stained well with Giemsa's stain. Definition was good and the organisms took on a purple colour similar to the chromatin of the leucocytes and platelets: this contrasted well with the pale pink staining of the erythrocytes. *E. parvum* occurred principally as small solid cocci at about the limit of optical resolution, or as slightly larger cocci with their centres staining less deeply than their periphery, giving the organisms a hollow appearance. Annular forms and a variety of other polymorphic forms were also seen. Most of the eperythrozoa were supracellularly placed on the erythrocytes, which were sometimes so heavily parasitized as to be more or less completely obscured by organisms. A few

eperythrozoa were seen in the spaces between the erythrocytes: these organisms were most numerous at the tails of smears. Polychromatic and other immature red cells were not frequently infected. Particles resembling eperythrozoa were sometimes seen in leucocytes, but *E. parvum* was not detected in cells or tissues apart from the blood. Fig. 1 illustrates a smear of blood infected with *E. parvum* to which *Staphylococcus aureus* was added to allow comparisons of size to be made.

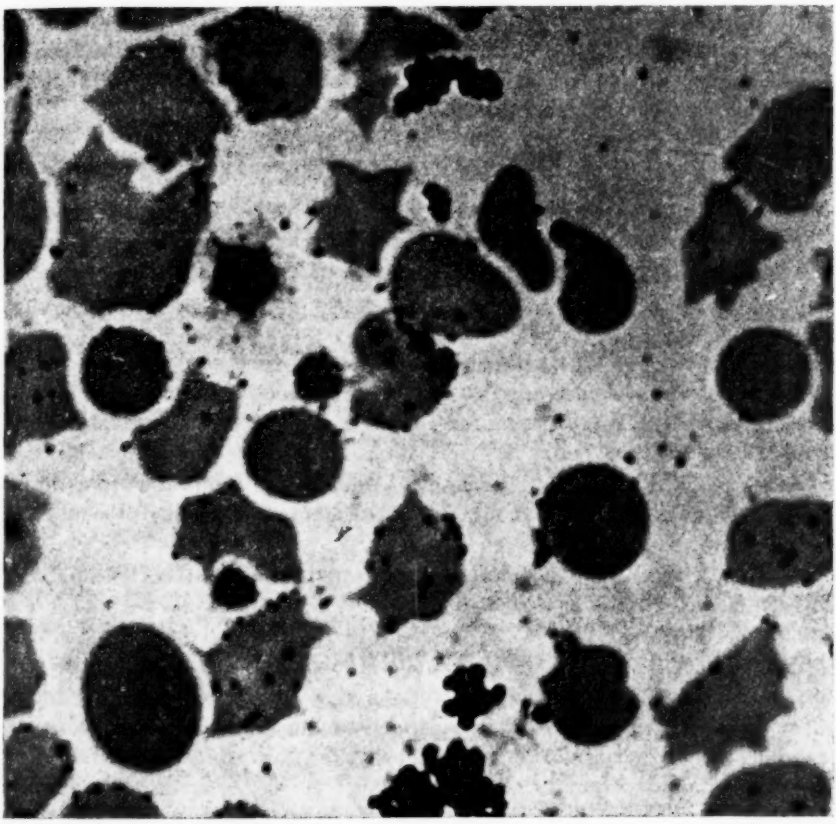

Fig. 1. Heavy infection of *Eperythrozoon parvum* in the blood of a splenectomized pig, with *Staphylococcus aureus*. × 2500.

In blood smears stained intravitally with cresyl blue and counterstained with Giemsa's solution *E. parvum* was compared with reticulocyte granules. The granules stained a deep dark blue, and their distribution and coarse appearance contrasted well with the distinct purple form of the eperythrozoa. *E. parvum* stained less well with Leishman's stain than with Giemsa's and poorly with 1% aqueous methylene blue and 0·1% aqueous basic fuchsin. The organisms took the counterstain only with Gram's, modified Ziehl-Neelsen's, Castaneda's and Macchiavello's stains.

Serum and plasma for filtration experiments were obtained from splenectomized pigs heavily infected with *E. parvum*. The cellular elements were removed by centrifugation and the infected materials were passed through gradocol membranes under negative pressure. The filtrates and the serum and plasma were protected from exposure to light. Seven pigs were inoculated with filtrates which had passed through membranes of average pore diameter (APD) not less than 0·57μ. Six of these pigs became infected with *E. parvum*. One pig which received a filtrate passed through a membrane of APD 0·41μ also became infected, but four pigs which received filtrates passed through membranes of APD 0·36μ or less, remained free of infection. Using the factor recently recommended by Black (1958), these results suggest a diameter of about 250 mμ for *E. parvum*.

(2) *Effect of increased and reduced temperatures*

Four samples of heparinized pig blood infected with *E. parvum* were incubated at 56, 37° C. and at room temperature, and the percentages of heavily and of lightly infected erythrocytes and of all infected erythrocytes were determined at intervals. In each case, the percentages of heavily infected erythrocytes (six or more eperythrozoa) decreased relatively quickly, and the persistence of infection was due to lightly infected erythrocytes (one to five eperythrozoa). The persisting organisms tended to lose their distinctive morphology, and the residual infection ultimately consisted of single, indistinct organisms, marginally placed on the erythrocytes. No eperythrozoa were detected in blood samples exposed to 56° C. for 20 min., and the infection had disappeared from three of four samples incubated at 37° C. for 12 hr. At room temperature visible organisms persisted for 7, 9, 10 and 13 days.

Twenty pigs which were inoculated with infected blood, serum or plasma, which had been stored at 5° C. for periods up to 96 hr., became infected with *E. parvum*. In one experiment, samples of infected and uninfected blood were sedimented by centrifugation. The plasma supernatants were exchanged and the erythrocytes were resuspended. After storage for 15 days at 5° C., 1 ml. of each of these suspensions was inoculated into a pig. Both pigs became infected with *E. parvum*.

Samples of heparinized and defibrinated blood and serum infected with *E. parvum* were stored for periods of 18 and 83 days at −30° C. After this time, 2 ml. of each sample were inoculated into susceptible pigs. All the pigs became infected with *E. parvum*. Sterile glycerol was added in a step-by-step fashion to two portions of heparinized blood to give final concentrations of 10 and 15 %, respectively. These samples, and one without glycerol, were cooled to −79° C. at the rate of 2–3° C./min. After a token storage period of 1 day, a sample of each was thawed, smears were made and pigs were inoculated. The smears of the blood containing glycerol closely resembled fresh blood smears containing *E. parvum*, but the blood without glycerol had almost completely haemolysed. Each blood sample was infective for pigs. After storage for 80 days, two pigs were inoculated with blood containing 15 % glycerol and two with blood only. All four pigs became infected with *E. parvum*.

(3) *Modes of transmission*

E. parvum was transmitted by the intravenous, subcutaneous and intraperitoneal inoculation of infected blood. Two pigs which received infective inocula by intratracheal injection also became infected. Heparinized blood heavily infected with *E. parvum* was discharged from a syringe into the throats of four pigs. Two pigs which received doses of 20 ml., and one pig which received 10 ml. blood became infected with *E. parvum*. A pig which received only 5 ml. blood did not become infected within an observation period of 29 days.

Seven pig lice (*Haematopinus suis*) were removed from a pig heavily infected with *E. parvum*. The lice were ground up in sterile normal saline, and 0·5 ml. of the resultant saline suspension was injected intravenously into each of two susceptible pigs. Both of these pigs became infected with *E. parvum* 18 days after the inoculation. In a second experiment, fifty lice were removed from two pigs heavily infected with *E. parvum*. Three ml. of a saline suspension prepared from twelve of these lice were injected intravenously into a susceptible pig, and the remaining living lice were transferred to two other pigs. The inoculated pig became infected with *E. parvum* after a prepatent period of 20 days. One pig to which living lice were transferred became infected 32 days later, but the second pig remained free of infection for 52 days.

(4) *Course of infection*

The prepatent periods of 103 primary infections of *E. parvum* in splenectomized pigs were observed on a thrice-weekly bleeding schedule. They ranged from 1 to 32 days, with a mean of 9·7 days. Sixteen prepatent periods were observed in intact pigs; they ranged from 2 to 13 days with a mean of 6·5 days. The distribution of prepatent periods according to length was similar in intact and splenectomized pigs. Patent infections ranged from 3 to at least 36 days in splenectomized pigs and from 3 to 29 days in intact pigs. No correlation was observed between the length of prepatent period and the length of the ensuing patent infection. Blood was taken from two splenectomized pigs 11 months after each had had a primary infection of *E. parvum*. Blood from one of these pigs infected a susceptible pig, but blood from the other was not infective. Blood was again taken from the carrier pig after a further 12 months (23 months after the primary infection): and it was again infective when inoculated into a susceptible pig.

The first sign of primary patent infections of *E. parvum* in splenectomized pigs was the appearance of one or two infected erythrocytes in blood smears. These erythrocytes were usually heavily infected with eperythrozoa. The numbers of eperythrozoa and of parasitized erythrocytes increased steadily, until at the peak of infection about 90 % of the erythrocytes were usually parasitized. The numbers of eperythrozoa then diminished gradually, and the numbers of parasitized erythrocytes also decreased, but more slowly. Eventually all traces of infection disappeared from blood smears, but recrudescences of parasitaemia usually occurred. When patent infections were observed in intact pigs, they followed a similar course, but the extent of the infection was always much less than that seen in splenectomized pigs.

After the peak of infection many splenectomized pigs developed an anaemia, from which some died. Normoblasts, megaloblasts, polychromasia, anisocytosis and macrocytosis were observed in blood smears from these animals. No specific lesions, apart from those of anaemia, were found at autopsy. No anaemic changes were detected in the blood smears taken from intact pigs infected with *E. parvum*. These observations were substantiated by the following experiment.

(5) *Production of anaemia in splenectomized pigs*

Five pigs from a litter of nine were weaned at 5 days old: they were splenectomized at 10 days old. The remaining four litter-mates were weaned at 11 days old: these pigs were left intact. At 13 days old two splenectomized and two intact pigs were inoculated intravenously with *E. parvum*. A third splenectomized pig was inoculated subcutaneously: on the eighteenth day of the experiment this pig was injected intravenously with neoarsphenamine. The pigs were examined haematologically before inoculation and thrice weekly thereafter.

The results are given in Tables 1 and 2. All the inoculated pigs became infected with *E. parvum* and all the controls remained free of infection. In splenectomized pigs 1 and 2 a severe anaemia followed the peak of infection. These pigs showed clinical signs of anaemia. They died on the sixteenth and seventeenth days of the experiment, and lesions of anaemia were found at autopsy. In splenectomized pig 3 the infection developed more slowly, and patent infection was removed by the treatment with neoarsphenamine. This pig did not become anaemic, but it died on the twenty-third day of the experiment. No lesions associated with anaemia were found at autopsy. The two control splenectomized pigs did not become anaemic. They died on the seventeenth and twenty-third days of the experiment and again no lesions of anaemia were found at post-mortem examination. The infected and control intact pigs remained healthy throughout the experiment.

(6) *Susceptibility to therapeutic agents*

The susceptibility of *E. parvum* to therapeutic agents was examined *in vivo*. Maximal doses of the drugs under test were used (Jones, 1954). The percentages of lightly and heavily infected erythrocytes in the peripheral blood were estimated before and at appropriate intervals after treatment.

Neoarsphenamine

Seven pigs were injected intravenously with a single dose of neoarsphenamine* at a dose rate of 40 mg./kg. body weight. In each pig a marked reduction in the numbers of eperythrozoa and in the numbers of infected erythrocytes occurred within 24 hr. of the treatment: in three pigs patent infection disappeared completely.

The possibility that neoarsphenamine could be used to sterilize infections of *E. parvum* was investigated. Ten splenectomized pigs were infected with *E. parvum* and paired according to weight and to the degree of infection in each. One member

* 'Novarsenobillon', May and Baker Ltd.

Table 1. *Haematological observations made during the course of infections of* Eperythrozoon parvum (*splenectomized piglets*)

Pig		Time on experiment (days)												
		0	4	6	8	11	13	15	16	17	18	20	22	23
(1) *E. parvum*, i/v, day 0	Degree of infection	.	+	+	+ + +	+ + +	+ + +	+ +	D	.	.	.	.	.
	RBC's 10^6/mm.^{3}	4·9	5·5	5·8	6·1	5·3	1·8	1·0	.	.	.	.	.	.
	Hb g./100 ml.	10·7	8·7	10·4	10·7	8·0	3·6	2·0	.	.	.	.	.	.
	Packed RBC volume	35	24	28	34	28	10	6	.	.	.	.	.	.
(2) *E. parvum*, i/v, day 0	Degree of infection	.	+	+ +	+ + +	+ + +	+ + +	+ + +	.	D	.	.	.	.
	RBC's 10^6/mm.^{3}	6·3	6·3	5·3	5·6	4·2	3·0	1·8	.	.	.	.	.	.
	Hb g./100 ml.	10·9	9·7	10·8	9·9	6·5	5·7	3·6	.	.	.	.	.	.
	Packed RBC volume	27	28	31	29	21	18	12	.	.	.	.	.	.
(3) *E. parvum*, s/c, day 0	Degree of infection	.	−	−	−	+	+ +	+ + +	.	.	+ + + N	−	−	D
	RBC's 10^6/mm.^{3}	5·4	6·0	5·8	4·8	5·4	5·2	5·1	.	.	5·2	5·6	5·7	.
	Hb g./100 ml.	8·9	10·8	10·3	9·2	8·1	10·5	9·4	.	.	8·7	8·4	6·1	.
	Packed RBC volume	36	34	31	25	25	29	28	.	.	17	24	17	.
(4) Control	Degree of infection	.	−	−	−	−	−	−	.	D	.	.	.	.
	RBC's 10^6/mm.^{3}	5·0	5·8	5·5	5·5	5·3	5·1	5·4	.	.	.	.	.	.
	Hb g./100 ml.	7·0	8·4	8·6	8·5	8·0	7·8	8·4	.	.	.	.	.	.
	Packed RBC volume	23	30	28	25	24	23	25	.	.	.	.	.	.
(5) Control	Degree of infection	.	−	−	−	−	−	−	.	.	−	−	−	D
	RBC's 10^6/mm.^{3}	6·1	5·5	4·8	7·0	9·3	5·5	5·1	.	.	5·0	4·9	6·1	.
	Hb g./100 ml.	11·1	10·5	8·3	10·3	9·7	10·9	8·4	.	.	8·8	8·8	8·3	.
	Packed RBC volume	29	27	35	29	27	28	21	.	.	28	28	24	.

D = died. N = neoarsphenamine. − Not infected + Lightly infected + + Moderately infected + + + Heavily infected.

Table 2. *Haematological observations made during the course of infections of* Eperythrozoon parvum (*intact piglets*)

Pig		Time on experiment (days) 0	4	6	8	11	13	15	18	20	22	25	27	29
(6) *E. parvum*, i/v, day 0	Degree of infection	.	+	+	++	+	+	−	++	++	++	++	−	−
	RBC's 10^6/mm.^{3}	8·1	6·3	7·9	6·0	6·2	6·0	5·3	6·3	5·6	7·0	6·2	5·7	5·6
	Hb g./100 ml.	14·1	11·8	11·0	10·8	9·4	9·9	7·8	9·2	8·8	10·4	11·1	9·5	11·1
	Packed RBC volume	40	35	35	31	29	27	21	27	26	30	31	28	29
(7) *E. parvum*, i/v, day 0	Degree of infection	.	+	+	+	+	+	−	+	+	+	+	−	−
	RBC's 10^6/mm.^{3}	5·3	6·5	6·5	5·5	5·8	5·8	5·5	6·0	5·8	6·9	4·2	4·5	7·2
	Hb g./100 ml.	10·5	10·3	9·7	7·4	8·5	8·3	8·5	9·2	8·3	8·4	9·1	8·1	9·4
	Packed RBC volume	32	35	32	27	23	16	24	23	24	33	25	22	30
(8) Control	Degree of infection	.	−	−	−	−	−	−	−	−	−	−	−	−
	RBC's 10^6/mm.^{3}	5·5	6·5	6·9	5·4	5·8	6·2	6·7	6·1	5·8	7·1	5·7	5·7	7·1
	Hb g./100 ml.	9·0	10·0	9·7	6·1	9·6	10·5	10·3	9·9	8·5	8·8	11·0	10·1	11·6
	Packed RBC volume	31	31	28	26	30	32	31	28	25	27	32	27	35
(9) Control	Degree of infection	.	−	−	−	−	−	−	−	−	−	−	−	−
	RBC's 10^6/mm.	7·2	5·7	6·1	6·6	6·6	6·2	5·0	6·1	7·7	7·4	6·2	7·0	7·6
	Hb g./100 ml.	13·3	9·6	11·1	7·5	8·6	9·4	8·5	9·4	9·0	10·0	9·9	10·2	11·0
	Packed RBC volume	34	31	29	29	26	24	25	29	25	33	29	30	32

− Not infected + Lightly infected ++ Moderately infected +++ Heavily infected.

of each pair received two intravenous injections of neoarsphenamine* with an interval of 24 hr. between them. The other pig in each pair received an equivalent volume of the vehicle in which the neoarsphenamine was suspended. Three pigs received neoarsphenamine at dose rates of 45 mg./kg. body weight, one 55 mg. and one 65 mg./kg. body weight. Blood smears were examined on the days of treatment and three times weekly thereafter. On the twenty-eighth day of the experiment four pigs which had received neoarsphenamine and one which had received vehicle were examined for the persistence of *E. parvum*. Blood from each pig was subinoculated into a susceptible pig and at the same time the donor pigs were re-inoculated with *E. parvum*.

The courses of the infections in the ten pigs are set out in Table 3. Patent infection disappeared from the five pigs which received neoarsphenamine, but it recurred in one pig 18 days after treatment. Patent infection continued in the pigs which received vehicle. Blood taken from three pigs which had received 45, 55 and 65 mg. neoarsphenamine per kg. body weight did not infect susceptible pigs with *E. parvum*. The three donor pigs developed patent infections following re-inoculation. Blood from one pig treated with 45 mg. neoarsphenamine per kg. body weight infected a susceptible pig. The donor pig developed a patent infection on re-inoculation. Blood from the pig which received vehicle infected a susceptible pig, but the donor failed to develop patent infection of *E. parvum* within 12 days of re-inoculation.

Sulphadimidine, penicillin and streptomycin

The susceptibility of *E. parvum* to sulphadimidine, penicillin and streptomycin *in vivo* was examined in the following experiments.

(1) Five pigs each received four intraperitoneal injections of sulphadimidine† at intervals of 24 hr. (Initial dose rate 0·1 mg./lb. body weight, maintenance dose rate 0·05 mg./lb. body weight.)

(2) Two pigs each received five intramuscular injections of 1 mega unit of crystalline sodium penicillin G‡ at intervals of 24 hr. (Pigs weighed 12·5 lb.)

(3) Four pigs each received three intramuscular injections of 1·5 mega units of procaine penicillin§ at intervals of 24 hr. An untreated control was kept. (Pigs weighed 13–29 lb.)

(4) Two groups of three pigs each received five intramuscular injections of streptomycin sulphate‖ at intervals of 24 hr. (Dose rate 10 mg./lb. body weight.) An untreated control was kept with each group.

No reductions in the numbers of eperythrozoa or of infected erythrocytes attributable to the treatment occurred in these experiments.

Oxytetracycline

The effect of oxytetracycline hydrochloride¶ upon *E. parvum in vitro* was

* 'Vetarsenobillon', May and Baker Ltd.
† 'Sulphamezathine', Imperial Chemical Industries (Pharmaceuticals) Ltd.
‡ May and Baker Ltd.
§ 'Distaquaine', Distillers Co. Biochemicals Ltd.
‖ Glaxo Laboratories Ltd.
¶ 'Terramycin', Pfizer Ltd.

Table 3. *The course of infections of* Eperythrozoon parvum *in ten splenectomized pigs before and after treatment with neoarsphenamine and neoarsphenamine vehicle, and after re-infection*

| No. of pig | Time on experiment (days) | | |

investigated in three experiments. Four infected pigs were treated with single intramuscular injections of oxytetracycline at dose rates of 5, 10, 20 and 43 mg./lb. body weight. An untreated control was kept. Twenty-four hours after treatment a marked reduction in the numbers of eperythrozoa and of infected erythrocytes had occurred in each of the treated pigs, but there was no reduction in the degree of parasitaemia in the untreated pig. Two infected pigs were treated at 24-hr. intervals with three injections of oxytetracycline at a dose rate of 5 mg./lb. body weight. The percentages of infected erythrocytes did not decrease until after the second injection. In the third experiment, five pigs were treated with two injections of oxytetracycline at a rate of 10 mg./lb. body weight, with an interval of 24 hr. between treatments. An untreated control was kept. The severity of the infection was reduced in each treated pig within 24 hr. of the first treatment, and a further reduction followed the second injection. The course of infection in the untreated pig remained unaltered.

IV. DISCUSSION

E. parvum has not been cultivated in the laboratory, and as the eperythrozoa are markedly host specific, all the experiments had therefore to be conducted in the natural host, the pig. Since experimental infections of *E. parvum* did not always become patent in intact pigs, the use of splenectomized animals was obligatory. The use of early-weaned piglets provided experimental animals comparable to other species of laboratory animals. However, for practical and economic reasons the correlation of experiments with the supply of pigs was difficult. This was particularly felt when attempts were made to cultivate *E. parvum*, in which it was necessary to test the infectivity of materials from the various passages. These attempts, and attempts to transmit *E. parvum* to mice, guinea-pigs and rabbits, were unsuccessful (Seamer, 1959).

The staining, morphology and location of *E. parvum* were characteristic of the genus *Eperythrozoon* (Weinman, 1944). While further evidence is required, the present results suggest a diameter of 250 mμ for *E. parvum*. Splitter (1952, 1953) reported that *E. suis* was retained by Seitz EK filters, and that *E. parvum* passed through them. *E. coccoides* passed through gradocol membranes of APD 0·36μ (Niven, Dick, Gledhill & Andrewes, 1952). *E. parvum* was retained by this size of filter in the present experiments.

There were few records in the literature of the viability of eperythrozoa after exposure to heat. Since the infectivity of the blood samples containing *E. parvum* was not tested in pigs after exposure to heat, the present results refer only to visible organisms and not to viable ones. The results do not suggest, however, that *E. parvum* is more resistant to heat than other species (Weinman, 1944; Thurston, 1955).

Although eperythrozoa and species of the closely related genus *Haemobartonella* have been preserved for short periods at temperatures close to 0° C. (Weinman, 1944; Thurston, 1955; Biberstein, Barr, Larrow & Roberts, 1956), only two records were found of their preservation at very low temperatures. Splitter (1952) preserved *E. suis* for at least 31 days at −32° C., and Kessler (1942)

preserved *H. muris* for at least 11 weeks at −79° C. Evidence has been produced to show that *E. parvum* can also be preserved at −30° C. and at −79° C. in pig blood. At the lower temperature *E. parvum* was also preserved in the presence of glycerol, which prevented haemolysis of the infected blood. Weinman & McAllister (1947) preserved many species of protozoa at −70° C. Polge & Soltys (1957) found that the survival of *Trypanosoma brucei* at −79° C. was enhanced by the presence of glycerol and a controlled rate of cooling. *E. coccoides* has also been preserved at −79° C. in mouse blood and in chick embryo tissues. The stability of this organism in chick embryo tissues may have been enhanced by the presence of 10 % sterile glycerol (Seamer, to be published).

Although eperythrozoa have been transmitted by parenteral injections, the evidence on their transmissibility by the oral route was conflicting. Weinman (1944) recorded that he had earlier failed to infect three splenectomized mice with *E. coccoides* given orally, but Thurston (1955) transmitted this organism to five of eight mice with infected blood given by mouth. Four pigs became infected with *E. parvum* after receiving infected blood by mouth in the present investigation. The oral route may be a natural channel for the transmission of eperythrozoa in certain species of host.

E. parvum was transmitted to four pigs by means of pig lice taken from infected pigs. These results, taken in conjunction with that of Jansen (1952), establish the pig louse (*Haematopinus suis*) as a potential vector of *E. parvum*. Eliot (1936) and Cannon & McClelland (1929) have shown that *E. coccoides* and *Haemobartonella muris* are also transmitted by lice. While Timmerman (cited by Weinman, 1944) has shown that the flea *Xenopsylla cheopis* may also transmit *H. muris*, other insect vectors of these organisms have not been recorded.

The lengths of the prepatent periods of infections of *E. parvum* in splenectomized and intact pigs varied considerably, but the mean prepatent periods were similar (9·7 days in splenectomized pigs and 6·5 days in intact pigs). Splitter (1953) recorded that the mean prepatent period obtained from twenty-one primary infections of *E. parvum* was 13·1 days.

E. parvum was shown to persist in one splenectomized pig for 23 months, but was eliminated from another within 11 months. No reports of the persistence of *E. parvum* were found in the literature. Splitter (1950*b*) found that a splenectomized pig remained a carrier of *E. suis* for 3 months, and McCluskie & Niven (1934) recorded the persistence of *E. coccoides* in splenectomized mice for 4 months. Weinman (1944) concluded that although intact rats infected with *H. muris* probably remained infected for life, the parasites were eventually eliminated by the splenectomized carrier. He believed that infections of eperythrozoa behaved similarly. Since Derrick, Pope, Chong, Carley & Lee (1954) found that intact mice eliminated *E. coccoides* in from 21 to 41 weeks it is apparent that the persistence of these organisms in intact and splenectomized hosts requires further investigation.

While intact pigs were apparently unaffected by infections of *E. parvum*, the organism frequently caused a severe and sometimes fatal anaemia in splenectomized pigs. However, in the experiment described, the deaths in the infected

splenectomized pigs could not be attributed to anaemia alone, since the control animals also died. Splitter (1952) reported that *E. parvum* was occasionally responsible for a disease in splenectomized pigs which resembled the ictero-anaemia of swine caused by *E. suis*. Weinman (1938) has shown that in splenectomized rats infected with *H. muris*, a haemolytic anaemia occurs, and that the organisms themselves are the haemolytic agents.

Sensitivity to organic arsenicals is a generic characteristic of the eperythrozoa. Splitter (1952) recorded that *E. parvum* was less susceptible to neoarsphenamine than *E. suis*: doses of less than 40 mg./kg. body weight were usually ineffective against *E. parvum*. Doses of 40 mg./kg. body weight were found to be effective in the present experiments. Infections of *E. parvum* in three pigs were sterilized by neoarsphenamine at dose rates of 45, 55 and 65 mg./kg. body weight, but it appears from the results that proof of sterilization may only be obtained by the subinoculation of blood from the treated animal.

E. parvum was susceptible to oxytetracycline hydrochloride *in vivo*. The minimum single effective dose, given intramuscularly, appeared to be 5–10 mg./lb. body weight. Oxytetracycline did not exert so marked an effect on *E. parvum* as did neoarsphenamine. Splitter & Castro (1957) found that *E. suis* was susceptible to treatment with tetracycline. *E. parvum* was not susceptible to sulphadimidine, penicillin or streptomycin under the conditions of these experiments. The susceptibility of *E. parvum* to the five therapeutic agents tested resembled the susceptibility of *E. coccoides* to these agents (Thurston, 1953).

V. SUMMARY

1. The staining and morphology of *E. parvum* were characteristic of the genus *Eperythrozoon*. The diameter of the organism was approximately 250 mμ.

2. Visible eperythrozoa disappeared in 20 min. from infected blood incubated at 56° C., and in 12 hr. at 37° C. They persisted for several days at room temperature.

3. *E. parvum* was preserved for short periods at 5° C. and for longer periods at −30 and −79° C.

4. Splenectomized early-weaned piglets were experimentally infected by the parenteral and oral routes, and by means of the pig louse *Haematopinus suis*.

5. The mean prepatent period of 103 primary infections in splenectomized pigs was 9·7 days. Patent infections sometimes persisted for at least 36 days. One pig remained a carrier of *E. parvum* for 23 months.

6. A severe anaemia occurred in splenectomized piglets affected with *E. parvum*, but intact piglets were apparently unaffected.

7. *E. parvum* was susceptible *in vivo* to neoarsphenamine and oxytetracycline, but not to sulphadimidine, penicillin or streptomycin.

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The reaction of some variants of *Ornithodoros moubata* Murray (Argasidae, Ixodoidea) to desiccation

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(*Received* 16 *April* 1959)

INTRODUCTION

In a series of publications (Walton, 1957, 1958*a*, *b*) evidence of a biological and epidemiological nature was presented in support of a hypothesis of biological variation within the species *Ornithodoros moubata*, the vector of African tick-borne relapsing fever. The existence of four variants was postulated in the East African area and these were designated forms A, B, C and D.

The possible existence of an additional variant collected in huts in South Africa was also mentioned. This form E has subsequently been shown to be distinct and was dealt with previously (Walton, 1959).

In the meantime, further information became available which showed that the wild form C from warthog burrows probably consisted of two variants. Form C was originally created after studying *O. moubata* from a warthog or porcupine burrow on Crescent Island, Lake Naivasha, in the Highlands of Kenya Colony (Heisch & Grainger, 1950). Evidence will be given in a later paper which shows that some wild strains appear to belong to a variant distinct from the Naivasha strain and are provisionally designated form F.

Examples of all these 'forms' were included in the present experiment, which was made to test ability to withstand desiccation.

THE STRAINS OF *ORNITHODOROS MOUBATA* USED IN THE EXPERIMENT

Strain A 1. *An East African domestic man-eating strain from a cool damp highland habitat*

This is the culture adopted as the type of form A and was obtained in a single African hut in a hillside village devoid of domestic fowls at an altitude of 6000 ft. and above Kangeta in the Jombeni Mountains of the Meru District of Kenya Colony (Walton, 1950).

Strain A 2. *A Central African domestic man-eating strain from a cool damp highland habitat*

Obtained from a single African hut from which domestic fowls were absent at an altitude of 5500 ft. at Murusagamba on the Ruanda–Urundi border in the Ngara District of Tanganyika.

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STRAIN A3. *An East African domestic man-eating strain from a cool damp highland habitat*

This strain of form A was obtained in a single African hut in the absence of domestic fowls at an altitude of 6000 ft. in the Othaiya Location of Nyeri District of Kenya Colony on the side of the Aberdare Mountains and on the opposite side of Mount Kenya from the location of strain A1.

STRAIN B1. *An East African chicken-eating domestic strain from a hot damp lowland habitat*

Strain B1 is the adopted type-strain of form B and was obtained from a single African hut at an altitude of approximately 50 ft. on the shore of the Indian Ocean at Tiwi in the Digo District of Kenya Colony south of Mombasa (Walton, 1955).

STRAIN C1. *A high-altitude East African wild warthog or porcupine burrow strain*

Strain C1 is the type-strain of form C and was originally collected by Mr W. E. Grainger in the burrow of a warthog or porcupine on Crescent Island in Lake Naivasha at an altitude of 6250 ft. in the Nakuru District of Kenya Colony (Heisch & Grainger, 1950).

STRAIN D1. *An East African domestic mixed-feeding strain from a hot dry habitat*

This is the type-strain of form D and was collected in a single African hut from which domestic fowls were absent at M'noho near Mwitikira in the Dodoma District of the Central Province of Tanganyika Territory at an altitude of approximately 3700 ft.

STRAIN E1. *A South African domestic strain*

Chosen as the type of form E, this strain was obtained from an African hut at Groot Marico in the Western Transvaal and was received from Dr Botha de Meillon.

STRAIN E2. *A South African domestic strain*

Another strain of form E obtained from an African hut at Lehututu in the central Kalahari Desert in Bechuanaland and received from Dr F. Zumpt.

STRAIN F1. *A low-altitude East African wild warthog burrow strain*

This strain, from a warthog burrow in the thorn thicket near Mwitikira in the Dodoma District of the Central Province of Tanganyika Territory, is the adopted type of form F.

HYBRID STRAIN B1 × F2. *A hybrid between an East African domestic chicken-eating strain and a warthog burrow strain derived from an African hut*

Strain F2 was derived from a mixed collection of *O. moubata* obtained from a single African hut from which domestic fowls were absent at Wuchichi at an

altitude of 5000 ft. in the Teita Hills of Teita District of south-east Kenya Colony, after prolonged starvation had eliminated all domestic forms. There are grounds for concluding that the survivors were introduced to the original tick population of the hut from outdoors, and could be assigned to the warthog form F (Walton, 1957, 1958*a*, *b*). A female of this Teita residue was crossed with males of strain B 1.

EXPERIMENTAL METHODS

Twenty females of each strain, unmated and not fed in the adult stage, all of which had reached maturity within a month of one another and were a random sample from the laboratory cultures, were placed in cut-down 3 in. by 1 in. thin glass specimen tubes fitted with copper gauze covers and placed together in a desiccator containing pure sulphuric acid. This desiccator was kept in a constant temperature room maintained at 32·2° C. (90° F.). The tubes containing the ticks were removed from the desiccator every third day and weighed, and were transferred to the balance in a second desiccator containing crushed potassium hydroxide in order to reduce sudden changes in temperature and humidity. This second desiccator was kept in the hot room. The temperature at the site of weighing varied from 72 to 78° F. and relative humidity from 45 to 75 %. Exposure to these values could not cause any detectable change in the weight of the ticks during the short time taken in making the weighings. Dead ticks were not removed and are included in the weight of the remaining living ticks in subsequent weighings. All solids, including excreta and the dust inevitably clinging to the ticks at the beginning of the experiment, were left in the tubes, and loss in weight is therefore equivalent to loss of water only. The experiment was continued until 100 % mortality was first reached in a strain and then discontinued, although 90 % of another strain were still living.

Table 1. *The final percentage weight loss, percentage survival and daily mean loss of weight of twenty female* Ornithodoros moubata *from nine strains, and one cross between different strains, when exposed to dry air at* 32·2° C. *for* 96 *days*

Strain	% weight loss (mg.)	% survival	Mean weight loss per tick per day (mg.)
A 1	55·4	60	0·29
A 2	73·6	5	0·40
A 3	53·6	65	0·31
B 1	73·6	0	0·30
D 1	63·8	35	0·35
C 1	66·4	5	0·31
F 1	54·9	35	0·31
E 1	42·0	90	0·15
E 2	36·7	75	0·17
B 1 × F 2	65·7	25	0·36

RESULTS

A. *Rate of water-loss*

Two rates of water-loss were found. All East African domestic and wild warthog or porcupine burrow strains lost water at a mean rate of 0·32 mg. per tick per day, while the South African domestic strains lost water at only half that rate, namely 0·16 mg. per tick per day (see Table 1).

Table 2. *The number of deaths recorded in the strains of* Ornithodoros moubata *at 3-day intervals when exposed to* R.H. 0% *and* 32·2° C., *observations starting on the 42nd day when the first deaths occurred, and ending on the 96th day when a* 100% *mortality was first reached in strain* B1

	A1	A2	A3	B1	D1	C1	F1	E1	E2	B1 × F2
42nd day	0	0	0	2	0	0	0	0	0	0
45th day	0	0	0	2	0	0	0	0	0	0
48th day	0	1	0	3	0	0	1	0	0	0
51st day	0	1	0	3	0	0	1	0	0	0
54th day	0	1	0	6	0	0	1	0	0	0
57th day	0	1	0	9	0	0	1	0	0	0
60th day	0	3	0	10	0	0	1	0	0	0
63rd day	0	4	0	10	1	1	1	0	0	0
66th day	1	5	0	10	1	1	1	0	0	0
69th day	1	7	0	11	3	2	1	0	0	0
72nd day	1	8	0	12	3	3	4	0	0	3
75th day	2	10	0	13	8	6	5	0	0	4
78th day	3	12	1	13	9	8	6	0	0	6
81st day	4	13	4	14	10	12	6	0	0	6
84th day	6	15	4	14	11	13	7	0	0	8
87th day	8	15	4	17	13	16	9	0	0	12
90th day	8	18	5	19	13	16	12	0	0	14
93rd day	8	18	5	19	13	16	12	0	1	15
96th day	8	19	7	20	13	19	13	2	5	15

Table 3. *The mean weight per tick in mg. in the strains of* Ornithodoros moubata *when entered to an atmosphere of* 0% R.H. *and* 32·2° C., *and subsequently at intervals of* 9 *days* (*the last interval was* 6 *days*)

	A1	A2	A3	B1	D1	C1	F1	E1	E2	B1 × F2
Initial weight	51·00	53·00	56·75	40·00	53·00	45·00	54·50	34·50	45·00	53·25
9th day	47·50	48·50	53·00	36·50	47·75	42·00	51·00	32·50	43·00	49·25
18th day	44·50	44·75	49·25	33·50	44·00	39·00	48·50	31·00	41·00	45·25
27th day	42·00	41·50	46·50	31·00	41·00	36·75	46·00	30·00	39·50	42·25
36th day	39·50	38·25	43·75	28·25	38·50	34·00	43·25	28·25	37·75	39·25
45th day	37·50	35·50	41·40	25·50	35·50	32·00	41·00	27·05	35·90	37·15
54th day	35·75	32·75	39·00	22·75	33·25	29·75	38·25	25·85	35·50	34·75
63rd day	33·35	29·35	37·00	18·85	30·65	27·80	36·35	24·65	33·80	32·45
72nd day	30·50	24·90	34·60	17·85	27·75	25·00	33·90	23·60	32·40	29·75
81st day	27·60	19·85	31·40	15·90	23·05	21·70	30·75	22·30	30·85	25·80
90th day	24·25	15·90	28·35	12·35	19·80	15·75	26·50	20·90	29·35	21·60
96th day	22·75	14·00	26·30	10·60	19·20	15·10	24·55	20·00	28·50	18·25

The initial mean weight and the subsequent mean weight per tick in each strain at 9-day intervals is given in Table 3. The differences are illustrated in Figs. 1 and 2.

B. *Survival rates*

The times of death of the ticks in each strain can be found from Table 2, and the percentages of survivors at the end of the experiment on the 96th day are given in Table 1.

Not only is it demonstrated that there are different rates of water-loss between, on the one hand, the South African domestic strains of *O. moubata* and, on the other hand, all other tested strains, but survival of a strain in either group is proportional to the initial mean weight of the strains.

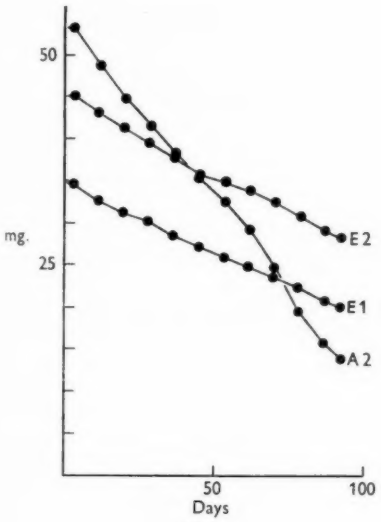

Fig. 1

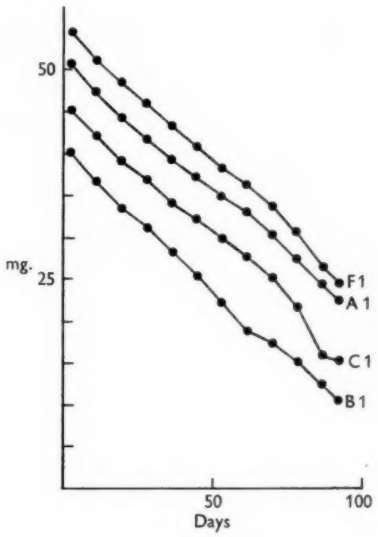

Fig. 2

Fig. 1. Mean weight loss per tick at 9-day intervals in twenty females of the *O. moubata* strains A 2, E 1 and E 2 when exposed to dry air at 32·2° C.

Fig. 2. Mean weight loss per tick at 9-day intervals in twenty females of the *O. moubata* strains A 1, B 1, C 1 and F 1, when exposed to dry air at 32·2° C. Strains A 3, D 1 and the cross between B 1 and F 2 are not shown. Reference to Table 3 will show that D 1 and the cross are identical while A 3 approximates closely with F 1.

DISCUSSION

The subject of water-loss and retention in *O. moubata* has been studied by Lees (1946, 1947, 1948) and Browning (1954*a*, *b*). Resistance to desiccation was shown to be of a high order and is obviously closely bound up with survival in nature. These workers also demonstrated the remarkable ability of these ticks (which group of *O. moubata* they used is uncertain) to secrete water into themselves from the atmosphere at relative humidity values of approximately 92% and over.

These characters allow the tick to survive for long periods between blood meals. Walton (1957, 1958*b*) also showed that the wild forms of *O. moubata* found in warthog or porcupine burrows were far more resistant to starvation than the East African domestic strains. Thus, 60 % of once-fed females of the East African domestic form A survived 9 months and the East African domestic form B survived 18 months, whereas form F (Daluni, Usambara Mountain, Tanga District, Tanganyika warthog or porcupine burrow strain (Walton, 1953)) showed a 60 % survival at 4 years and 8 months when kept at a relative humidity of 85 %.* Comparable information on the South African domestic strains is not likely to be available for some years.

The similarity between the rates of water-loss in the East African domestic strains and the wild warthog burrow strains (which are now known to have a distribution extending southwards into the area occupied by the South African domestic form E) is a result that could have been expected in view of the similarity between the micro-climates discovered in their respective habitats by Geigy & Mooser (1955) and Walton (1957, 1958*b*, p. 115). Apparently no measurements of micro-climate have been made in South Africa comparable with those made in East Africa, nor is it known whether form E is a true domestic tick or a recent invader of human habitations from some wild habitat as yet imperfectly known. The tortoise might conceivably have been the original host (Bedford, 1924).

The largest of the East African strains of domestic *O. moubata* (strain A3) could be estimated from the present results to survive up to 155 days in dry air; the smallest (B1) only 95 days. The smallest South African domestic strain tested (E1) could survive for about 155 days and the largest (E2) for 200 days.

The rather high mean loss of weight per day (0·4 mg.) obtained in the Central African domestic strain A2 from the Ruanda–Urundi border with Tanganyika cannot be entirely ignored merely as experimental error, since this strain is known to differ from the East African strains in a number of minor biological characters. Presumably this is a Congo variant of Form A on the fringe of its distribution and a rate of water-loss of 0·4 mg. per day might be expected in a variant centred in a high-humidity region.

Strain D1, the mixed-feeder from African huts in the arid and hot centre of Tanganyika, lost water at the rate of 0·35 mg. per day, which is somewhat unexpected and its resistance to starvation would be worth investigating. The similarity between the rate of water-loss in strain D1 and in the hybrid strain need not be regarded as indicating hybridization in strain D1. Evidence to be published elsewhere suggests that strain D1 is unlikely to be a hybrid between form B and form F or hybridized with form F, although these forms do occur in quite close proximity in Central Tanganyika.

Initial weight of a strain of *O. moubata* has a profound effect upon the length of survival, and results simply from the rate of water-loss. Size of individuals of a strain could be regarded as more of an adaptation possessed by a form to withstand variations of climate within its distribution rather than a positive form

* The survivors of this protracted experiment died on an air journey from Tanganyika to London in June 1958.

character. The difference in initial weight between strains E 1 and E 2 would allow the latter strain from the Kalahari desert to survive in dry air some 2 months longer than the former. In a period of exceptional drought and in the absence of food this would be likely to assure the survival of the strain of larger individuals while eliminating the smaller.

SUMMARY

1. Ten strains of *Ornithodoros moubata* not fed as adults and never mated were exposed to dry air at 32·2° C. and the effect recorded by measuring the loss of weight and survival rates.

2. Two rates of water-loss were found and *O. moubata* may be divided into three groups on physiological grounds. Strains found in native huts in South Africa differ from all other tested strains by losing water in dry air at 0·16 mg. per day. All other tested strains lost water at 0·32 mg. (range 0·29–0·4 mg.) per day. The wild strains of the East African warthog and porcupine burrows are distinguished from the East African domestic strains by their ability to withstand prolonged starvation.

3. The initial mean weight of a strain in any of the groups has a considerable effect upon survival in dry air, the percentage survival (in 100 days tested) being directly proportional to the original weight of the ticks. Size of individuals could alone lead to ultimate survival differences of two months in different strains of the same form.

I especially wish to thank Professor E. Holmes for generous laboratory facilities at the East African Institute for Medical Research, Mwanza, Tanganyika; Dr Botha de Meillon and Dr F. Zumpt of the South African Institute for Medical Research for so kindly supplying the South African *O. moubata*; Mr K. L. Cockings, technical assistant, for making the constant-temperature room and for building up the cultures of *O. moubata*; Mr E. Benton for the thermostatic connexions, and Mr Nashon Ndale for his painstaking maintenance work in the laboratory. Professor D. S. Bertram and Professor P. C. C. Garnham have very kindly read the manuscript.

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Geckobia japonicum, a re-publication of *Pterygosoma japonicum* Kishida, 1927. (Acarina, Pterygosomidae)

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(*Received* 18 *April* 1959)

In a literature search for species of *Pterygosoma* it has been my good fortune to find a figure and Japanese description of *Pterygosoma japonicum* Kishida, 1927, in *Figuraro de Japanaj, Bestej* Tokyo, 1927, Hokurykan and Co. Ltd. This species has not been listed by the Zoological Record, and indeed it seems to have been completely overlooked in the West. Hence this re-publication.

I am deeply indebted to Dr Hajime Kamo, Assistant Professor in the Department of Pathology, Faculty of Medicine, Kyushu University, Fukuoka, Japan, for sending photostat copies of the figure and also for the translation of the description which are reproduced here. The translation has been modified slightly, and so mistakes are my responsibility.

Translation of the type description

Female. The body length is 400μ, its breadth 300μ. The body is densely covered with stout plumose setae. There is a chitinous plate on the dorsal and on the ventral surface of the body. The ventral or sternal plate is nearly rectangular in outline. The gnathosoma has large palpi (Fig. 1, 3); the small palpal thumb has many spines.

The chelicerae consist of two segments; the first one is long while the distal one is scissor-like. The genital and anal openings lie at the end of the body. There are four pairs of legs, each with a pair of coxal setae.

Distribution: in Honshu, Shikoku, and Kyushu Islands, Japan.

Parasitic on a gecko.

The body colour in life is a beautiful red.

It is clear that this species is not a *Pterygosoma* for a number of reasons. Thus the number of ventral body setae is atypical (*Pterygosoma* with three pairs, though *P.* (*Gerrhosaurobia*) *hystrix* Lawrence, 1935, has numerous ventral body setae, these are simple and restricted to the lateral parts of the venter). The body shape, the demarcation between coxae I–II and III–IV and the juxtaposition of coxae II and III are all different from *Pterygosoma*. The presence of a ventral shield is quite unknown in this genus. The short plumose setae on the legs and the similar seta on pedipalp segment II is characteristic of *Geckobia* rather than *Pterygosoma*. The form of the pedipalp apex is quite peculiar.

It is unfortunate that no dorsal view is given since most of the taxonomic characters of the Pterygosomidae are dorsal in position. However, most of the characters cited above are reconcilable with the genus *Geckobia* Megnin, 1878. Support is given to this conclusion by reason of the host being a gecko, a family of lizards which are characteristically parasitized by the genus *Geckobia*.

This species differs from all species of *Geckobia* known to date, though it bears some resemblance to *G. oedurae* Lawrence, 1936, from Southern Rhodesia.

Certain features of the drawing appear peculiar, but in the absence of specimens they are ignored for the present. Thus, for example, it is impossible to know what reliance to place on the sternal shield structure. It may be a pronounced chitinous structure or, on the other hand, it may simply denote a lack of the body striations so characteristic of the scale-mites.

Thus it is proposed to designate *Pterygosoma japonicum* Kishida, 1927, as *Geckobia japonicum* (Kishida), 1927.

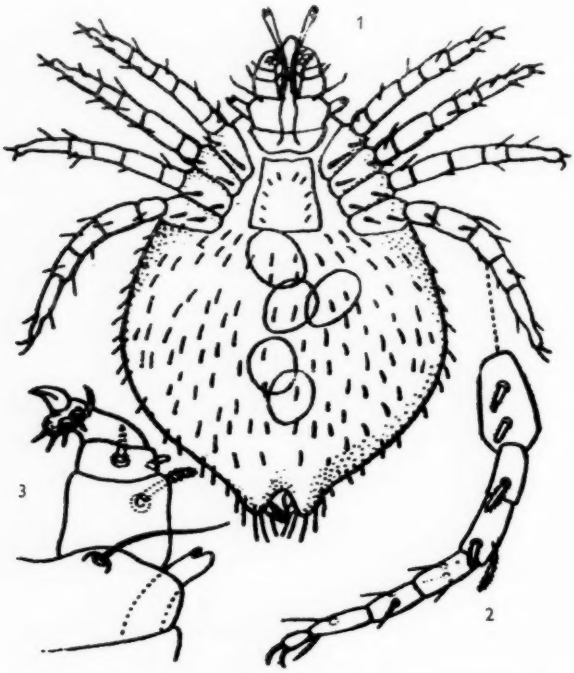

Fig. 1. Photostat copy of the original figure of *Geckobia japonicum* (Kishida), 1927, from the unchanged 1942 edition of *Figuraro de Japanaj Bestej*, Tokyo, 1927. Hokurykan and Co. Ltd. 1, Female, ventral view; 2, left, leg IV; 3, left, palp and pseudostigmatic organ.

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Observations on the natural enemies of *Meligethes aeneus* (F.) and *M. viridescens* (F.) [Coleoptera: Nitidulidae]

By PHILIP OSBORNE

Edinburgh and East of Scotland College of Agriculture, Edinburgh

(*Received* 5 *May* 1959)

INTRODUCTION

The incidence of natural enemies of *Meligethes aeneus* (F.) and *M. viridescens* (F.) has been recorded by several authors, particularly in Germany, but little attention has been paid to the morphology and development of the post-embryonic stages. With the greater interest shown in recent years towards the relationships between host and parasite, and the importance of natural enemies in exercising a controlling influence upon the fluctuations of pest populations, it is considered timely to present the results of biological and morphological observations as a basis for further study in the evaluation of the parasite complex of these economically important beetles.

The only previous work on post-embryonic stages of a *Meligethes* parasite was published by Friederichs (1921), who described and illustrated the adults, egg and larval stages of what he considered to be *Isurgus heterocerus* Thoms. Apart from this the incidence only of parasites and predators is recorded, together with observations on the biology of the adults.

Records of natural enemies

Meligethes aeneus

Parasites

Isurgus (*Thersilochus*) *morionellus* (Holmgr.) [Hym: Ichneumonidae] Brischke (1880), Oberstein (1919), Miles (1927).

Isurgus (*Thersilochus*) *heterocerus* (Thoms.), Börner & Blunck (1920), Friederichs (1921), Wolff & Krausse (1926).

Thersilochus sp. [Hymn: Ichneumonidae].

Diospilus capito (Nees) [Hym: Braconidae], Börner (1942), Osborne (1955).

Diospilus oleraceus Hal., Börner and Blunck (1920), Speyer (1921).

**Microgaster* sp. [Hym: Braconidae], Bogdanov-Katkov (1920).

Cryptoserphus parvulus (Nees) [Hym: Proctotrupidae], Osborne (1955).

Zeteticontus sp. [Hym: Encyrtidae], Osborne (1955).

Predators

Coccinella septempunctata [Col: Coccinellidae], Friederichs (1921).

Malachius bipustulatus [Col: Malachiidae], Friederichs (1921).

Hemerobius sp. larvae [Neuroptera: Hemerobiidae], Börner & Blunck (1920).

Theridium impressum [Araneida], (Wolff & Krausse, 1926).

* This is almost certainly an error according to G. J. Kerrich.

Meligethes viridescens

Callimone difficilis Nees [Hym: Torymidae] (Laboulbene, 1877).
Isurgus sp. (Osborne, 1955).
Isurgus heterocerus (Thoms.).
Thersilochus sp.
Diospilus capito (Nees) (Osborne, 1955).
Cryptoserphus parvulus (Nees) (Osborne, 1955).
Zeteticontus sp.

All the recorded hymenopterous parasites are internal parasites of the host larvae; parasitization of the adult host has not been observed.

The present paper describes and illustrates for the first time the post-embryonic stages of hymenopterous endoparasites found inhabiting the larvae of *Meligethes aeneus* and *M. viridescens* in the east of Scotland, and includes observations on the bionomics and morphology of the adult parasites.

MATERIALS AND METHOD

Samples of eggs and larvae of *M. aeneus* and *M. viridescens* were collected from *Sinapis arvensis* L. inflorescences at South Queensferry, West Lothian, every 3 days in 1955 from June until September. These were dissected and all stages of parasites were recorded and photographed. The parasite eggs were related to the parasite first instars by observing the developing larvae within the eggs. The last instars were related to the adults which subsequently emerged, and the eggs were related to the adults by dissection of the ovaries. This technique was necessary to determine the relationship between eggs, larvae and adults because of the existence of multiple parasitism. The intermediate instars between the first and last of each species were not distinguishable from each other morphologically and so are not described or figured.

Diospilus capito (Nees)

Synonymy: see Marshall (1889).

Hosts: *Meligethes aeneus* (F.) (Börner, 1942).
Ceuthorrhynchus leprieuri Bris. (Börner, 1942).
Psylliodes chrysocephala (L.) (Meuche, 1940).
Bytiscus betulae (L.) (Feytaud, 1918).
Meligethes viridescens (F.) (Osborne, 1955).

Egg (Fig. 1A–E)

Minute, transparent, colourless, narrowly ovate, widest equatorially with a pedicel at one end (*pd*). Soon after oviposition the egg swells, becomes lemon-shaped (Fig. 1B) and later almost spherical (Fig. 1C). As the egg continues to increase in size the pedicel is incorporated with the stretched chorion (Fig. 1D). In several instances eggs were found with the pedicels embedded in the fore- or hind-gut wall of the host but this was not invariable.

Eggs recovered from the host varied in size from 0·15 mm. long by 0·08 mm.

wide to 0·35–0·4 mm. in diameter before hatching. The embryo develops within a trophamnion (*tr*) consisting of a single layer of cells lining the chorion (Fig. 1D and E).

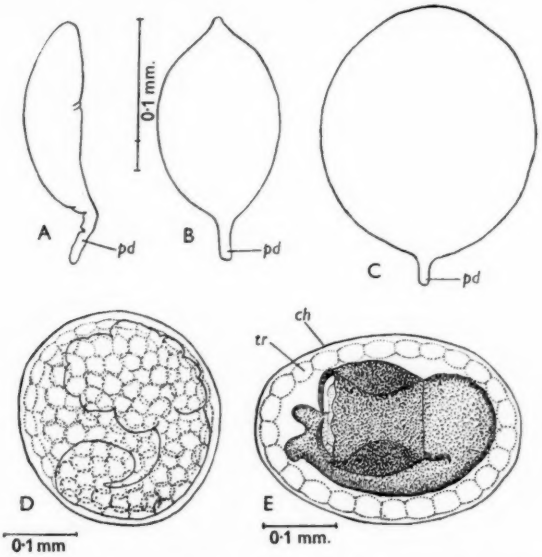

Fig. 1. *Diospilus capito* (Nees) egg: A, removed from ovary; B, after oviposition; C, some time after oviposition; D, containing developing embryo within the trophamnion; E, just prior to hatching. *ch*, chorion; *pd*, pedicel; *tr*, trophamnion.

First instar (Fig. 2)

The first instar can be seen at an advanced state of development, curled up within the trophamnion (Fig. 1E). Before emergence the head capsule darkens to brownish black and the mandibles, which are capable of movement at this stage, pierce the trophamnion. Upon emergence the larva maintains a C-shaped curve and frequently executes sudden bending movement, pushing the head ventrally towards the hind end, then slowly straightening (Fig. 2C).

The head-capsule (Fig. 3) is black, heavily sclerotized, flattened dorso-ventrally, widest anteriorly and constricted towards the base. It is in the form of an incomplete ring covering the entire dorsal surface (*dw*) and curving ventro-laterally to enclose the sides of the head (*lw*). The ventral surface, except laterally (*vw*), is unsclerotized. Long, sharply pointed falcate mandibles (*m*) articulate with the anterior angles of the head-capsule. A pigmented projection (*p*) occurs anteromedially from the unsclerotized part of the head between two pairs of lateral fleshy tubercles; one conical pair (*cp*) arising dorsally and a rounded pair (*rp*) ventrally.

The body is colourless, semi-transparent and consists of thirteen segments. In very young in stars (Fig. 2A) the segments narrow towards the hind end but in older larvae (Fig. 2B) they are approximately equal in width so that the body is

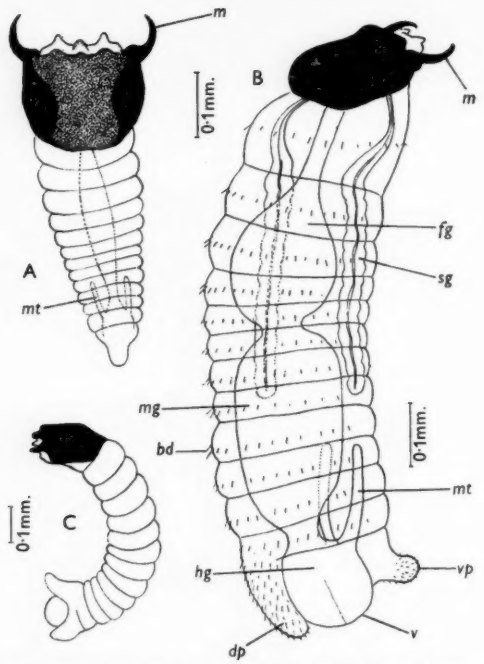

Fig. 2. *Diospilus capito* (Nees): A, newly emerged first-instar larva (dorsal); B, mature first-instar larva (dorso-lateral, semi-diagrammatic); C, immature first-instar larva (lateral). *bd*, backwardly directed microspines; *dp*, dorsal fleshy lobe; *fg*, foregut; *hg*, hindgut; *m*, falcate mandibles; *mg*, midgut; *mt*, Malpighian tubule; *sg*, salivary gland; *v*, external vesicle; *vp*, ventral fleshy lobe.

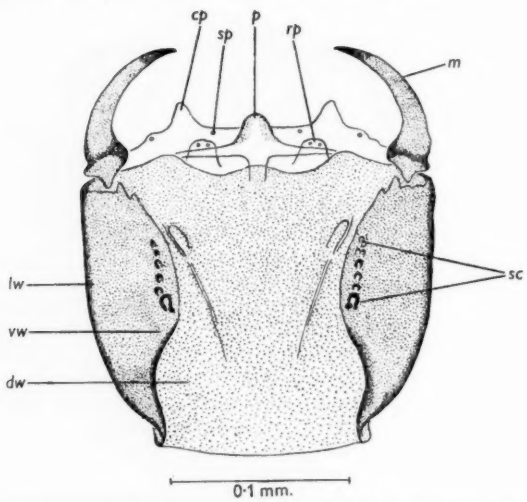

Fig. 3. *Diospilus capito* (Nees): head-capsule of first-instar larva (ventral). *cp*, dorsal conical fleshy tubercle; *dw*, dorsal wall; *lw*, lateral wall; *m*, left falcate mandible; *p*, pigmented projection; *rp*, ventral rounded fleshy tubercle; *sc*, irregularly sclerotized pattern; *sp*, papilla; *vw*, ventral wall.

almost cylindrical. The last segment is characteristically shaped, forming a dorsal (*dp*) and a ventral (*vp*) fleshy lobe between which is a large, hemispherical external vesicle or evagination of the proctodaeum (*v*). In older first instars a transverse row of short, backwardly directed spines (*bd*) may be seen dorsally and laterally on each segment, while similar spines cover the surface of the fleshy lobes on the last segment. Spiracles are absent.

A pair of long salivary glands (*sg*) and a well-defined fore-gut (*fg*) and mid-gut (*mg*) may be seen internally through the transparent integument. Two narrow blind tubes (*mt*) arise ventrally from the wall of the hind-gut (*hg*) and project into the body cavity where they probably function as simple Malpighian tubules.

It is the black head-capsule of this instar which is easily seen through the cuticle of the host as a black spot, thus betraying its presence. Friederichs (1921) gives an illustration of a first-instar larval parasite which he assumed to be the first instar of *Isurgus heterocerus*, but a comparison of his illustration ('plate II, fig. 5') shows clearly that he was mistaken and that his figure is of the first instar of *Diospilus capito*.

Last larval instar (Fig. 4)

This stage is hymenopteriform, vesiculate, creamy-white in colour, consisting of a head and thirteen well-defined segments with a pair of spiracles (*sp*) laterally on all segments except the pro- and metathorax and anal segments. The three thoracic, first three and last three abdominal segments are covered dorsally with numerous minute cuticular spines or microtrichia (*cs*) giving the dorsal surface, at the anterior and posterior ends, a pilose appearance. Laterally, abdominal segments 4–7 have similar cuticular spines. Longer setae (*b*) are found dorsally and in a transverse row on the thoracic and first two or three abdominal segments. The head-capsule (*h*) is well-defined, mainly unsclerotized but with a definite structure including weak mandibles, antennal disks, labial and maxillary papillae, sclerotized facial struts and setae (Fig. 5). The terminology of Short (1952) has been adopted in Fig. 5.

When the host larva enters the soil to pupate the *Diospilus* larva is still in an intermediate instar which continues to feed internally until almost filling the host's skin and then moults to the last larval instar. At this stage it bursts through the host's skin, head first, on the ventral surface and wriggles out. While still half-emerged it continues to suck the remaining juices by applying its mouth to the exterior of its host. When the host is little more than a dried dead skin the *Diospilus* larva spins a cocoon in the cavity in the soil prepared by its host for pupation. It is probably this cocoon which was observed by Faber, Fischer & Kalt (1920) when they described *Meligethes aeneus* as pupating within a cocoon.

The cocoon consists of two separate layers; an outer layer of loosely woven yellow silken threads, and an inner, paper-like layer composed of yellow silken threads fastened together with a clear cement. The external measurements averaged 3·0 mm. long by 1·75 mm. wide.

After 10–11 days adult *Diospilus capito* (determined by G. E. J. Nixon) emerged contemporaneously with *Meligethes* adults developed from unparasitized larvae

which had entered the soil to pupate at the same time as the parasitized larvae. The reared *D. capito* did not breed in captivity, but the earliest bred individuals emerged in July when there were still numerous *Meligethes* larvae in the field: thus it is possible that there may be more than one generation of *D. capito* a year.

The question of alternate hosts recorded in the literature was not investigated.

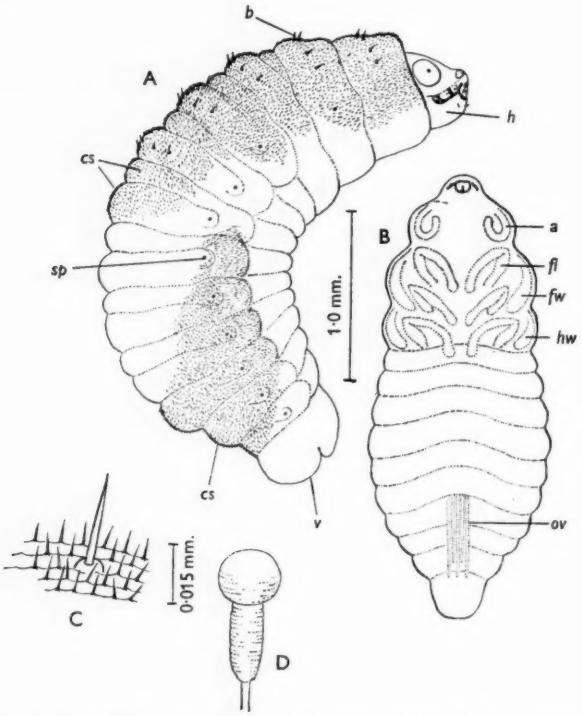

Fig. 4. *Diospilus capito* (Nees): A, final instar larva (lateral); B, prepupa (ventral); C, detail of cuticle from dorsal region of prothorax showing a seta and microtrichia; D, mesothoracic spiracle (lateral). *a*, imaginal antenna; *b*, seta; *cs*, cuticular spines or microtrichia; *fl*, imaginal foreleg; *fw*, imaginal forewing; *h*, head-capsule; *hw*, imaginal hindwing; *ov*, imaginal ovipositor; *sp*, right spiracle on 4th abdominal segment; *v*, vesicle.

Isurgus spp.

During the present study two species of *Isurgus* were found parasitizing *Meligethes aeneus* and *M. viridescens*. One was identified by G. J. Kerrich as *Isurgus heterocerus* (Thoms.). The other was not identified to species level, so for convenience it will be referred to in this paper as *Isurgus* sp. A.

In the Continental literature Brischke (1880) and Oberstein (1919) mention *Thersilochus morionellus* as a parasite of *Meligethes aeneus*. According to Schmiedeknecht (1911) the genus *Thersilochus* has been divided into several genera and *T. morionellus* Holmgr. is now *Isurgus morionellus* (Holm.).

Börner & Blunck (1920) described the differences between the antennae and

ovipositors of *Isurgus morionellus* and *I. heterocerus*. From their descriptions it will be shown that the parasite they described as *I. morionellus* resembles very closely *Isurgus* sp. A and is probably identical with it.

Isurgus heterocerus (Thoms.)

Synonymy: see Schmiedeknecht (1911)

Egg (*Fig.* 6)

Spindle-shaped, more strongly curved dorsally, more pointed towards the hind end. Chorion dark brown to black but slightly less dark at the extremities; smooth and without spines. When present within the body cavity of a *Meligethes* larva it is clearly visible through the integument as a dark spot.

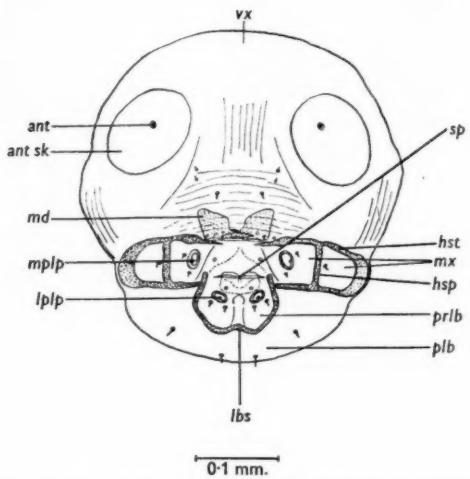

Fig. 5. *Diospilus capito* (Nees): head-capsule of final instar (anterior). *ant*, antenna; *ant sk*, antennal socket; *hsp*, sclerotic spur of hypostoma; *hst*, hypostoma; *lbs*, labial sclerite; *lplp*, labial palp; *md*, right mandible; *mplp*, maxillary palp; *mx*, left maxilla; *plb*, post-labium; *prlb*, pre-labium; *sp*, silk press; *vx*, vertex. (Nomenclature after Short (1952).)

First instar (Fig. 6C, D and E)

This stage emerges from the egg only when the host is fully fed and about to enter the soil to pupate. The anterior end of the egg-shell splits transversely on the ventral surface as the larva emerges and the cap thus formed remains attached to the egg-shell (Fig. 6B). The larva partially emerges head first, leaving the hind end of the body still enclosed by the egg-shell, remaining in this position throughout the first instar.

The first instar resembles that of *Isurgus* sp. A except (*a*) the head is more strongly sclerotized, (*b*) each segment is strongly sclerotized posteriorly (Fig. 6D), giving the body the appearance of alternating pale and dark yellowish bands, (*c*) the segmentation is so strongly pronounced in the earlier part of the stadium

that the body appears serrate in outline, and (*d*) for the presence of minute papillae (*mp*) on the ventral and lateral surfaces of the head (Fig. 7) and a pair of minute papillae, laterally, on each thoracic segment and the first six abdominal segments (Fig. 6E).

Last instar larva

Attempts to isolate the last instar were not successful because, in most cases, *I. heterocerus* was not the only parasite present in the host larvae examined and as a result of the competition it failed to develop. In some cases eggs failed to hatch

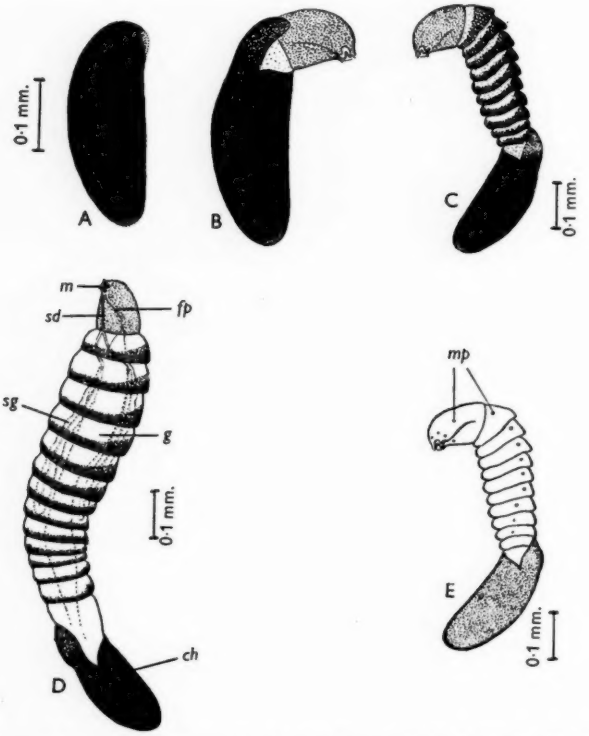

Fig. 6. *Isurgus heterocerus* (Thoms.): A, egg from ovary (lateral); B, first-instar larva hatching from the egg (lateral); C, first-instar larva with caudal end retained within the egg-shell; D, older first-instar larva; E, young first instar (lateral) showing papillae on head and body. *ch*, pigmented chorion; *fp*, sclerotized floor of the pharynx; *g*, gut; *m*, mandible; *mp*, papillae; *sd*, salivary duct; *sg*, salivary gland.

even in the absence of other parasites, or when hatched the first-instar larvae died. Numerous examples were encountered of overwintered *Meligethes* adults with one to three black egg-shells embedded in the fat-body showing that some *Meligethes* larvae parasitized by *Isurgus heterocerus* were capable of completing their development. This may indicate some resistance on the part of the host or it may be that *I. heterocerus* is a facultative rather than an obligate parasite of *Meligethes*.

Isurgus sp. A

Egg (Fig. 8A)

Colourless, subcylindrical, or almost parallel sided, equally rounded at both ends and slightly curved along its whole length. The chorion is densely covered with minute slender spines. There is no trophamnion development. When newly deposited in the host's body it measures 0·25 mm. long but swells to 0·5 mm. long before hatching.

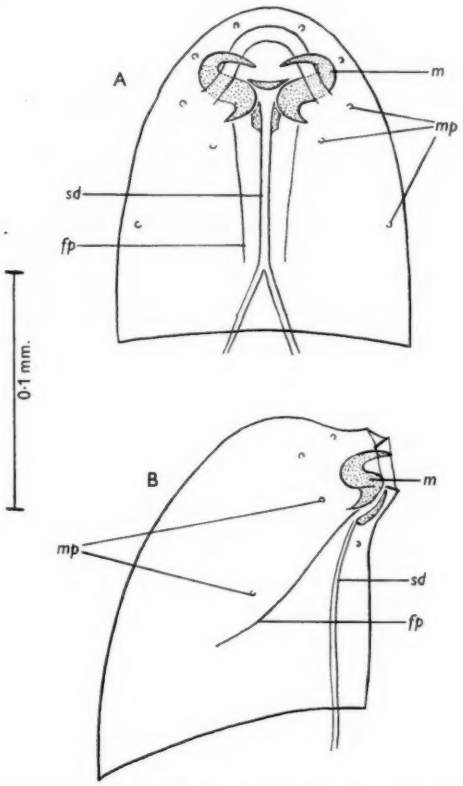

Fig. 7. *Isurgus heterocerus* (Thoms.): head-capsule of first-instar larva. A, ventral; B, lateral. *fp*, sclerotized floor of pharynx; *m*, mandible; *mp*, papillae; *sd*, salivary duct.

First instar (Figs. 8, 9)

This is a caudate-type larva with a small, somewhat bullet-shaped head (*hc*) which is slightly sclerotized but unpigmented, strongly curved dorsally and equipped with a pair of short, strongly falcate mandibles (*m*) anteriorly on the ventral surface. The floor of the pharynx (*fp*) is sclerotized and is seen in lateral view as a thin, highly refractive line. Paired salivary glands (*sg*) are connected by a common salivary duct (*sd*) opening just posterior to the mandibles. Muscles (*ms*) were observed to raise and lower the roof of the pharynx to create suction during feeding.

Segmentation of the body is not well defined even in the youngest stage. The cuticle is smooth and without papillae, tubercles or spines. Spiracles are absent.

Within the host the larva executes alternate bending and stretching movements, pushing the head ventrally towards the caudal end and then straightening.

Last larval instar (Fig. 10)

This stage is hymenopteriform but not vesiculate; creamy-white, with thirteen well-defined segments and a head, closely resembling the last instar of *Diospilus*

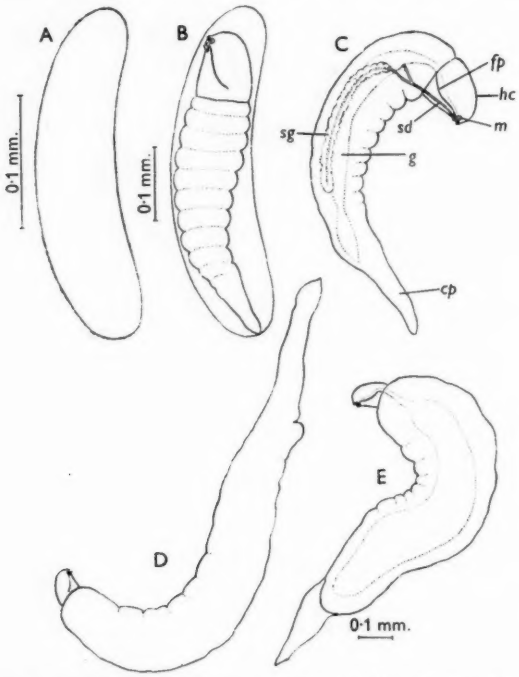

Fig. 8. *Isurgus* sp. A: A, egg; B, egg containing developing first-instar larva; C, young first-instar larva (lateral); D, older first-instar larva (lateral); E, first-instar larva about to moult (lateral). *cp*, caudal process; *fp*, sclerotized floor of pharynx; *g*, gut; *hc*, head-capsule; *m*, mandible; *sd*, salivary duct; *sg*, salivary gland.

capito. The cuticle, however, is tuberculate (Fig. 10C) except for the transversely oval smooth areas (*dc*) without tubercles on the dorsal surfaces of abdominal segments 1–5. Ventrally there is a pair of smooth circular areas (*tc*) on each thoracic segment overlying the imaginal disks of the legs. Each segment has a transverse row of bristles dorsally, and two bristles laterally except for the prothorax where there are three. Dorsally, on the abdominal segments 8–10, the tubercles are more pointed, spinelike and directed posteriorly (Fig. 10B). Spiracles (*sp*) are present on the mesothorax and abdominal segments 1–9.

The head is well defined and very similar to that of *D. capito* except for the more robust, short, heavily sclerotized mandibles and differences in the sclerotization

of the facial struts (Fig. 11), particularly the labial sclerite (*lbs*) which is incomplete ventrally.

As in *D. capito* the final larval instar is formed after the host enters the soil to pupate. Within a few days of the host entering the soil the intermediate stages of *Isurgus* sp. A develop rapidly and consume almost the entire body contents of the host before moulting to the final instar. The final instar then breaks through the host's integument and completes its feeding externally before spinning a cocoon

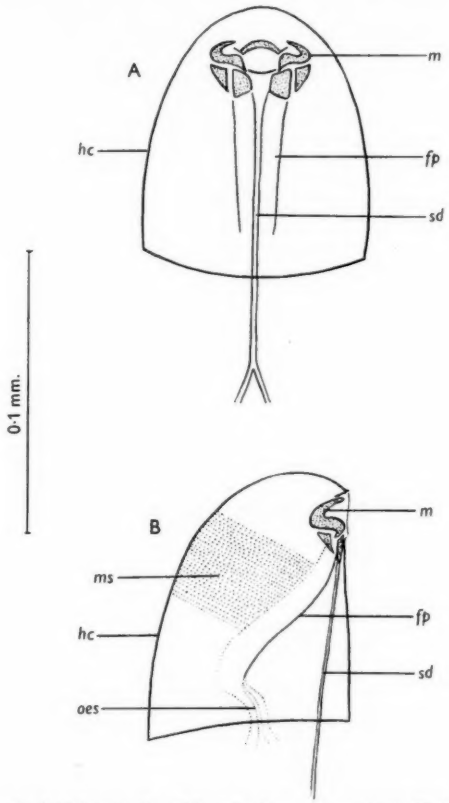

Fig. 9. *Isurgus* sp. A: head-capsule of first instar. A, ventral; B, lateral. *fp*, sclerotized floor of pharynx; *hc*, head-capsule; *m*, mandible; *ms*, muscle controlling sucking action of pharynx; *oes*, oesophagus; *sd*, salivary duct.

in which to pupate. Unlike *D. capito*, the adult *Isurgus* sp. A does not emerge about 10 days later but remains within the cocoon until the following spring in a state of diapause. Cocoons examined in November showed the adults completely formed and alive but inactive. The cocoon is different from that of *Diospilus capito*. It is white and consists of two layers of silken threads, both of which have a paper-like consistency imparted to them by a covering of transparent cement. It is slightly larger than a *D. capito* cocoon, measuring 3·2 mm. long by 2 mm. wide.

It is not possible to tell by external examination whether a *Meligethes* larva is parasitized by *Isurgus* sp. A since none of the immature forms is pigmented. In many cases, however, minute reddish spots may be seen on the integument of the host. These are probably the scars of wounds inflicted by the parasite during oviposition. The reddish spots were also found on larvae parasitized by *Diospilus capito* and *Isurgus heterocerus*.

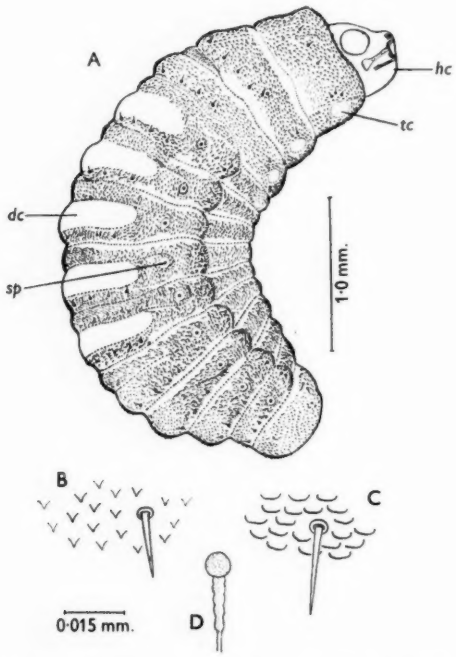

Fig. 10. *Isurgus* sp. A: A, final instar larva (lateral); B, detail of cuticle from dorsal region of 8th–10th abdominal segments, showing a seta and sharply pointed tubercles; C, detail of cuticle from dorsal region of the prothorax showing a seta and rounded tubercles; D, mesothoracic spiracle. *dc*, smooth areas without tubercles; *hc*, head-capsule; *sp*, spiracle on 4th abdominal segment; *tc*, smooth circular areas.

Morphological differences between the females of Isurgus *sp. A and* I. heterocerus *Isurgus* sp. A

Ovipositor (Fig. 16B): longer than in *I. heterocerus*, slender throughout its length; the basal half almost straight while the apical half curves gently upward, the curve increasing towards the sharply pointed tip. On the dorsal surface there is a small notch about one-twelfth of the length from the tip. It is free from the abdomen for nearly two-thirds of its length.

Antennae (Fig. 12A): first flagellar segment (fs_1) $2\frac{1}{2}$–3 times as long as broad and slightly longer than the second. Sensillum basiconicum on each of flagellar segments 4–6, hook-like, long and curved.

Head: less transverse with temples less narrowed behind the eyes.

Propodeum (Fig. 13A): longer and narrower; ratio of length to width 3:1.

Isurgus heterocerus

Ovipositor (Fig. 16C): shorter and stouter, almost straight but gently curving upward and becoming half as wide again about one-fifth of its length from the tip. About one-seventh of the length from the tip, on the dorsal surface, there is a more pronounced notch than in *Isurgus* sp. A. It is free from the abdomen for half its length.

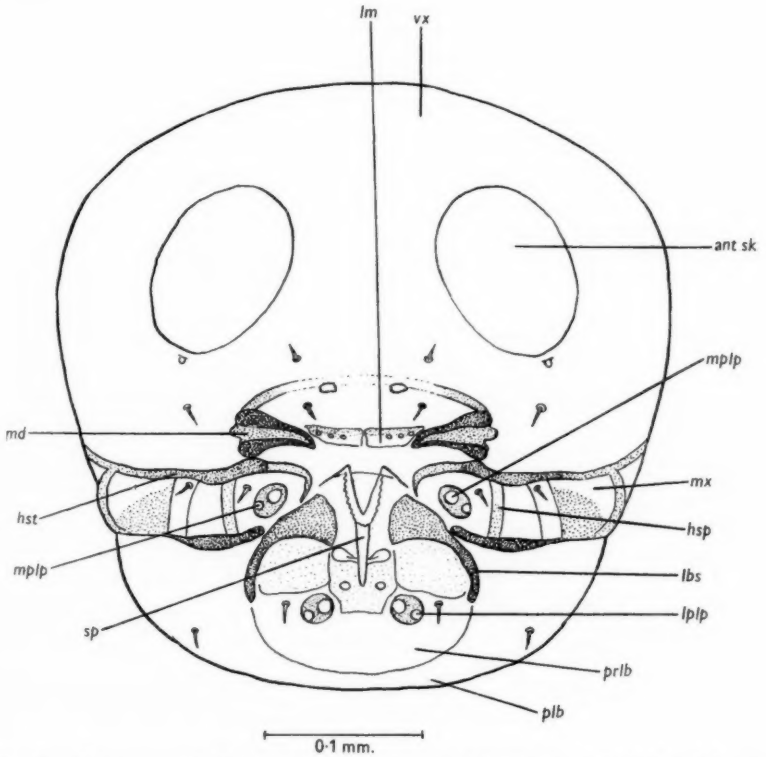

Fig. 11. *Isurgus* sp. A: head-capsule of last larval instar (anterior). *lm*, labrum. (For explanation of other lettering see Fig. 5).

Antennae (Fig. 12D): first flagellar segment about twice as long as broad and obviously longer than the second. Sensillum basiconicum on each of flagellar segments 4–6, peg-like, shorter, almost conical and less curved.

Head: more transverse with temples more narrowed behind the eyes.

Propodeum: (Fig. 13B): shorter and wider; ratio of length to width 2:1.

Literature and discussion (Isurgus *spp.*)

Börner & Blunck (1920) differentiate the two *Isurgus* spp. according to the size relationship of the '4th and 5th antennal segments', the two segments being similar in size and shape in *I. morionellus* while in *I. heterocerus* the 5th segment is 'clearly shorter and somewhat thicker than the 4th'. Since they did not differentiate between the scape and flagellum of the antennae their 4th and 5th segments may be assumed to be equivalent to the 1st and 2nd flagellar segments in my description, in which case *Isurgus* sp. A is probably the same species as that described by Börner & Blunck as *I. morionellus*.

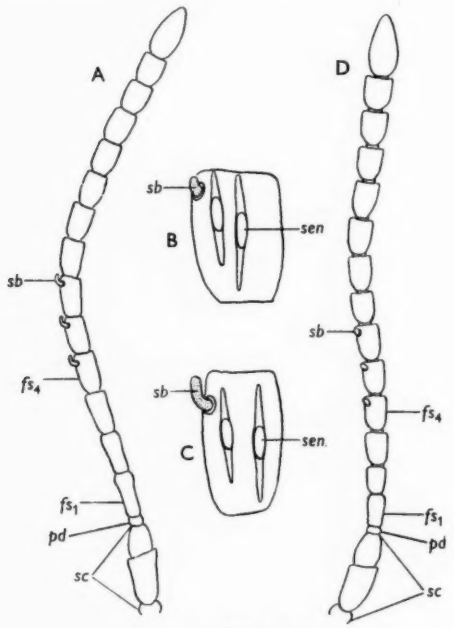

Fig. 12

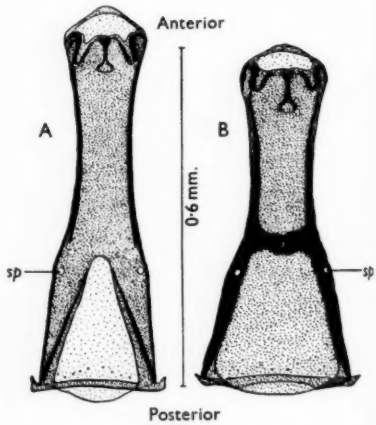

Fig. 13

Fig. 12. Comparison of the antennae of *Isurgus* sp. A and *I. heterocerus* (Thoms.) (setae and sensilla placodea omitted). A, left antenna of *Isurgus* sp. A; B, 6th flagellar segment of *Isurgus* sp. A; C, 6th flagellar segment of *I. heterocerus* (Thoms.); D, left antenna of *I. heterocerus*. fs_1, first flagellar segment; fs_4, fourth flagellar segment; *pd*, pedicel; *sb*, sensillum basiconicum; *sc*, scape; *sen*, sensillum placodeum.

Fig. 13. Comparison of propodia of *Isurgus* sp. A and *I. heterocerus* (Thoms.). A, *Isurgus* sp. A; B, *I. heterocerus*. *sp*, spiracle.

Thersilochus sp.

A female parasite found ovipositing in the buds of *Sinapis arvensis* was identified by G. J. Kerrich as a species of *Thersilochus*. Little is known about the immature stages of this genus, apart from the work of Cushman (1916) who described the biology and figured the development of *T. conotracheli* Riley, and Beirne (1941) who figured the head-capsule of the last instar of an unnamed species. Börner &

Blunck (1920) illustrate the ovipositor of an unidentified parasite which closely resembles the ovipositor of my specimen (Fig. 16D).

The present work does not add very much to existing knowledge, but it has been discovered that this parasite lays its eggs in the eggs and first instars of its host indirectly through the bud walls. The egg (Fig. 14A) resembles that of *Isurgus* sp. A but it is longer, more pointed posteriorly and is not pilose. The first instar (Fig. 14B) is very similar to that of *Isurgus* sp. A but the integument is strongly tuberculate and transversely wrinkled, particularly on the dorsal surface, as compared with the comparatively smooth integument of *Isurgus* sp. A.

Female *Thersilochus* sp., fully formed, alive but inactive, were found on 7 October 1957, in cocoons from soil containing parasitized *Meligethes aeneus* larvae collected on 24 June 1957.

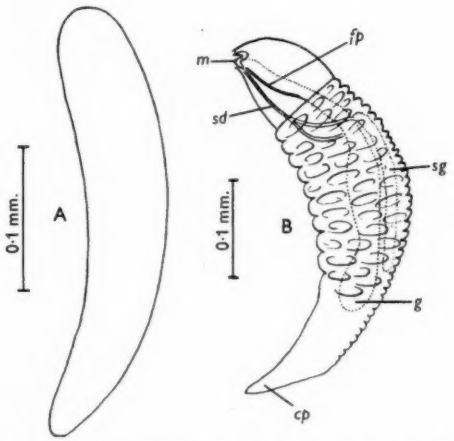

Fig. 14. *Thersilochus* sp. A, egg from ovary; B, newly hatched first-instar larva. *cp*, caudal process; *fp*, sclerotized floor of pharynx; *g*, gut; *m*, mandible; *sd*, salivary duct; *sg*, salivary gland.

Cryptoserphus parvulus (Nees)

Synonymy: *Codrus parvulus* Nees (1834).
Proctotrupes parvulus Haliday (1839).
Serphus parvulus André (1907).
Cryptoserphus parvulus Kieffer (André, 1907).
Cryptoserphus parvulus (Nees), Kieffer (1914).

Descriptions of the male and female are given by Kieffer (1914) and Nixon (1938). Morley (1922) mentions *Cryptoserphus parvulus* (Nees) as having been bred from *Boletus* fungi in Germany; from *Orchesia micans* (Col. Serropalpidae) and *Diphyllus lunatus* (Col. Erotylidae).

Egg (Fig. 15B, C)

Ovate, colourless, without a pedicel, chorion smooth. Eggs removed from the ovary are approximately 0·28 mm. long by 0·21 mm. wide. In the host's body

cavity they increase in size to 0·4 mm. long by 0·18 mm. wide. The embryo develops within a trophamnion (*tr*) consisting of a single layer of cells lining the chorion.

First instar (Fig. 15A, D)

Prior to emergence the larva (Fig. 15C) lies with its caudal process (*cp*) bent upwards and forwards dorsally towards the head, but on hatching it straightens out. This stage is mandibulate and polypodeiform. The head-capsule (*hc*) is

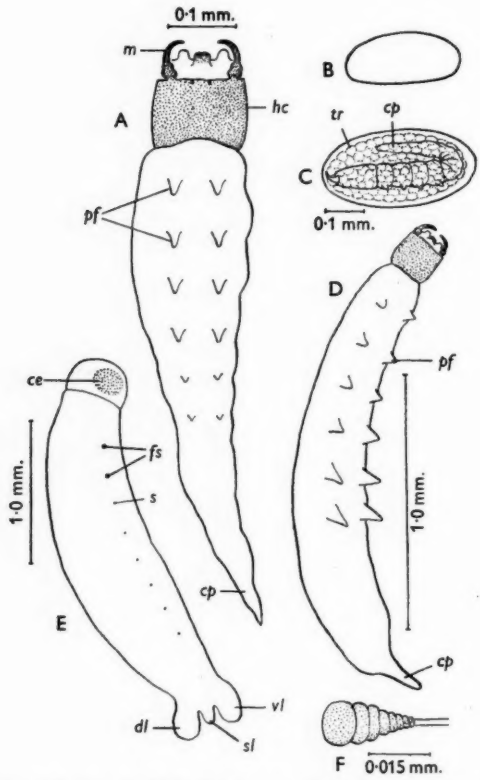

Fig. 15. *Cryptoserphus parvulus* (Nees). A, young first-instar larva (ventral); B, egg; *C*, egg dissected from host and containing a fully developed embryo within a trophamnion; D, older first-instar larva (latero-ventral); E, final instar larva (lateral); F, functional spiracle from the thoracic region. *ce*, imaginal compound eye; *cp*, caudal process; *dl*, dorsal fleshy lobe; *fs*, functional spiracles; *hc*, head-capsule; *m*, mandible; *pf*, ventral fleshy processes; *s*, non-functional spiracle; *sl*, median fleshy lobe; *tr*, trophamnion; *vl*, ventral fleshy lobe.

sclerotized, yellowish, flattened dorso-ventrally and completely surrounds the head, unlike that of *D. capito*.

The mandibles (*m*) are long, falcate and sharply pointed. The body of the young first instar is transparent and colourless, tapers posteriorly, and the segmentation

is weakly defined. The three thoracic segments and the first three abdominal segments each have a pair of fleshy processes (*pf*) on the ventral surface. Older first instars are more cylindrical and the fleshy processes on the abdominal segments increase in size until they are about twice the size of the thoracic processes. Spiracles are absent.

Last larval instar (Fig. 15E)

This stage is sacciform and not fundamentally different from the intermediate instars except for the presence of a pair of functional spiracles (*fs*) on the meso- and metathorax, and a pair of non-functional spiracles (*s*) on each of the first six abdominal segments. It is creamy-white with a smooth cuticle. There are no microspines or setae. The head is globular and devoid of sclerotization. Facial struts are rudimentary and the mandibles are small and weak. Imaginal compound eyes (*ce*) are visible as two faintly pinkish areas through the transparent cuticle of the head. The caudal segment has two large fleshy lobes posteriorly, one of which is dorsal (*dl*) and the other ventral (*vl*) with a smaller lobe (*sl*) between them.

Unlike the two species of *Isurgus* and *Diospilus capito*, the last larval instar completes its feeding internally and does not emerge entirely from its host but remains with the hind end of the abdomen embedded in its host's body. In every case observed, the *Cryptoserphus* larva broke through the host's cuticle head first on the ventral surface towards the hind end and wriggled out until it came to lie venter to venter with its host. It remained in this position and pupated with its posterior abdominal segments still embedded in its host and without spinning a cocoon. After about 10 days in the pupal state, adults emerged and mated almost immediately and proceeded to oviposit in host larvae exposed to them.

Oviposition and development

Cryptoserphus parvulus was the only parasite studied which oviposited readily in captivity. The ovipositor is very short and protected by heavily sclerotized gonapophyses (Nixon, 1938). The hind part of the abdomen is extremely flexible and extensile and can execute intricate movements such as doubling back upon itself at an angle of 180°. When searching for hosts the female locates a *Meligethes* larva with her antennae and then feverishly stabs at it with her ovipositor which she inserts in and out of the open flower at high speed.

Observations made on the behaviour of a single pair and the development of their progeny showed that under laboratory conditions the eggs hatched in 4 days; the first instar lasted 6 days; the intermediate stage 3 days; the last larval instar 5–6 days; and the pupal stage 9–10 days; making a total of 27–29 days from egg to adult. It was also noted that (i) host eggs were not attacked, (ii) both 1st and 2nd instars of the host are suitable for oviposition, (iii) more than one egg may be laid in a host but it is not certain whether the host is repeatedly attacked or whether a number of eggs is introduced during one act of oviposition, (iv) only one parasite per host completes its development, and (v) more than one generation per annum is possible.

Zeteticontus sp.

Data on this parasite are very limited. Specimens bred from *Meligethes aeneus* and *M. viridescens* were sent to G. J. Kerrich who identified them as belonging to the genus *Zeteticontus* but the species is undescribed and new to Great Britain. The genus is described by Silvestri (1915) and Mercet (1921).

Nothing is known about the immature stages. Its presence was discovered when a *Meligethes aeneus* larva taken on 7 July 1953 was found by 23 July to have swollen and taken on the appearance of a reddish brown dipterous puparium. Inside was a hymenopterous pupa. Several *Meligethes* larvae in a similar condition yielded parasites which were sent to the British Museum where they were retained. It is hoped that a description of this new species will be published at a later date.

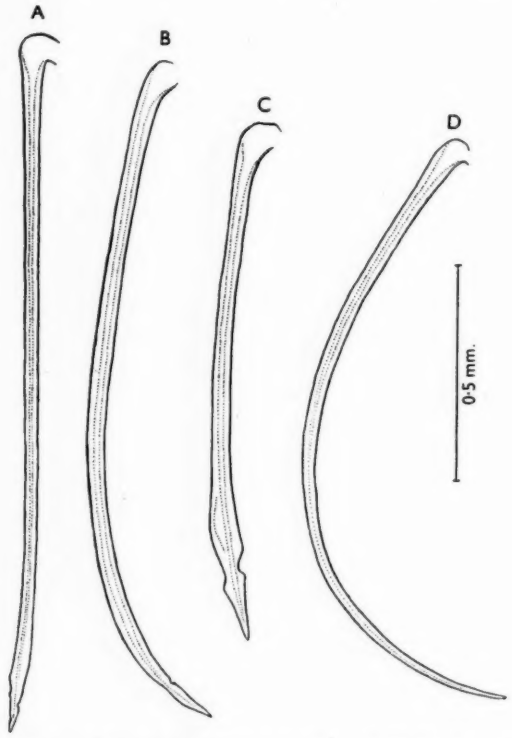

Fig. 16. Ovipositors of hymenopterous parasites of *Meligethes aeneus* (F.) and *M. viridescens* (F.) (from the left side). A, *Diospilus capito* (Nees); B, *Isurgus* sp. A; C, *I. heterocerus* (Thoms.); D, *Thersilochus* sp.

Protozoa *Organisms inhabiting adult* Meligethes

Overwintered *M. aeneus* adults taken on the flowers of volunteer turnips at Seton Mains, East Lothian, on 20 May 1955, and then dissected, were found to have large numbers of protozoa in the mid-gut. These were identified as belonging to

the order Gregarinidae, sub-order Eugregarinina, tribe Cephalina of the class Sporozoa, and were in the sporont stage exhibiting the association known as 'syzygy'.

Nematoda

On 12 May 1952, two female *M. aeneus* were found to have a large unidentified nematode in the body cavity.

Acarina

On two separate occasions a female *M. aeneus* was found with an unidentified large bright-red larval mite attached to the dorsal surface of the abdomen beneath the elytra.

SUMMARY

The recorded parasites and predators of *Meligethes aeneus* (F.) and *M. viridescens* (F.) [Coleoptera: Nitidulidae] are given together with descriptions and illustrations, for the first time, of the post-embryonic stages of the hymenopterous parasites *Diospilus capito* (Nees) [Braconidae], *Isurgus heterocerus* (Thoms.) [Ichneumonidae], *Isurgus* sp. A, and *Cryptoserphus parvulus* (Nees) [Proctotrupidae]. The egg and first instar of *Thersilochus* sp. are described and figured, and a species of *Zeteticontus* mentioned. The antennae, propodea, and ovipositors of the two species of *Isurgus* are compared and the ovipositors of *Diospilus capito*, *Isurgus* sp. A, *I. heterocerus* and *Thersilochus* sp. are illustrated.

A protozoan (Gregarine), a larval mite and a nematode, none of which has been identified, are recorded inhabiting *Meligethes* adults.

I would like to express my thanks to Mr G. J. Kerrich, Mr G. E. J. Nixon and Dr M. W. R. de V. Graham for their determinations of material sent to them on numerous occasions; to Mr G. J. Kerrich for reading the manuscript and offering suggestions for its improvement; and to Mr J. Lane who assisted with the collection of material.

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Tetrahymena chironomi sp.nov., a ciliate from midge larvae, and the current status of facultative parasitism in the genus *Tetrahymena**

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(*Received* 12 *May* 1959)

CONTENTS

I. Introduction 111
II. Materials and methods of investigation 113
III. Morphological description of *Tetrahymena chironomi* 115
IV. Sexuality in *Tetrahymena chironomi* 119
V. Systematic status of *Tetrahymena* species, particularly those exhibiting facultative parasitism 122
VI. Consideration of host–parasite relationships 138
A. General considerations 138
B. *Tetrahymena chironomi* and *Chironomus plumosus* 139
C. Other species of *Tetrahymena* and their hosts 144
VII. Summary 147
References 149

I. INTRODUCTION

A number of naturally occurring instances of facultative parasitism involving ciliated protozoa very likely assignable to the holotrich hymenostome genus *Tetrahymena* have been reported in the literature of the past 40 years. These cases are of considerable interest from the point of view of the systematic status of the protozoan species involved and, perhaps of greater importance, from the aspect of the possible significance of the relationship between the several hosts and their symbionts. Whether the invasion of the host is accidental, temporary, or of regular occurrence in the life cycle of the ciliate, such problems as mode of entry, degree of pathogenicity, and reciprocal effects on host and parasite need to be elucidated and studied from a comparative approach.

The present paper has two principal aims: to describe the morphology, systematics, and general bionomics of a new species of *Tetrahymena*, the thirteenth assignable to this growing genus of hymenostome ciliates and the eighth to be found in association with some other organism as host; and to treat the problems just mentioned concerning the systematics and host–parasite relationships of all tetrahymenid species which, to date, have been involved to any extent in accidental

* Much of this work was carried out by the author in 1951–52 as an Atomic Energy Commission post-doctoral research fellow in the laboratories of Professor E. Fauré-Fremiet, Collège de France, Paris. Augmentation of the original data, analysis and inclusion of the more recent literature, and additional indispensable studies on related species have been made possible with the aid of grants from the National Science Foundation.

or facultative parasitism. Although much more work, especially of an experimental nature, needs to be carried out, it is of prerequisite importance to consider the past findings first and to re-evaluate and re-interpret the data in the literature in the light of our present knowledge on the subject.

Three groups of published works are pertinent to the present investigation. It is interesting to note that over half of these publications have appeared within the past 5 or 6 years. The first group is composed of papers reporting cases in which insects, notably larvae of mosquitoes and midges, have served in nature as hosts for *Tetrahymena*-like ciliates. These works include: de Boissezon (1930); Corliss (1952*b*, *c*, 1953*a*, 1954*a*), Ghosh (1925), Grassé & de Boissezon (1929), Keilin (1921), Lamborn (1921), MacArthur (1922), Muspratt (1945, 1947), Treillard & Lwoff (1924), and Wenyon (1926).

The second group of publications includes recordings of the natural or accidental occurrence of presumably congeneric ciliates in the bodies of vertebrates or of invertebrates other than insects. Some are only very incidentally concerned with problems of facultative parasitism; in fact, a few may represent only cases of histophagous forms attacking moribund invertebrates as scavengers. As is also true for some of the works cited in both the preceding and following paragraphs, the protozoa involved may have been identified under a variety of names other than *Tetrahymena*. The investigations include those of van den Berghe (1934), Borden (1948, unpublished), Burns *et al.* (1955), Canella (1954), Corliss (1951, 1952*a*, *c*, 1953*a*, 1954*a*), Dobrzańska (1958), Epstein (1926), Gellért (1956, 1957), Kahl (1931), Kazubski (1958), Knight & McDougle (1944), Kozloff (1946, 1956*b*, *c*, 1957), Nance (1954), Nigrelli, Jakowska & Padnos (1955, 1956), Penard (1922), Seamster (1952), Shumway (1940), Speidel (1951–58), Stout (1954), Thompson (1955*a*, 1958*a*, *b*), Thompson & Speidel (1955), Warren (1932), and Windsor (1959).

Finally, of considerable value is the small group of works which are in large part experimental in nature. In these papers may be found reports concerning successful or unsuccessful attempts at laboratory infection of a variety of hosts, including insect larvae, with known strains of *Tetrahymena* species. Such experimentation has been carried out by Janda & Jírovec (1937), Kozloff (1956*a*–*c*), Lwoff (1924), McCaul (1956), McLaughlin (1959), Muspratt (1945, 1947), Nigrelli, Jakowska & Padnos (1956), Speidel (1951, 1953, and others), Thompson (1955*b*, 1958*a*, *b*), and Watson (1946).

The tetrahymenid organism described as a new species in the present paper was discovered repeatedly and in great numbers in the body cavity of midge (chironomid) larvae examined in Paris during the year 1951–52*. Without doubt it is

* Actually the first discovery was purely accidental. Following the routine practice in Fauré-Fremiet's laboratory, I was separating out the intestinal tract of larval *C. plumosus* to use as food for cultures of several kinds of hymenostome ciliates. This operation is carried out on the stage of a low-power stereoscopic microscope and I happened to notice a number of ciliates in the remains of a dissected larva. Concentrated checking of hundreds of additional larvae, over a period of some months, resulted in the finding of not only the species described here as new, but also an amicronucleate strain of *T. pyriformis*, as already reported briefly elsewhere (Corliss, 1952*b*, 1953*a*) and considered again in appropriate sections of the present paper.

identical with the ciliates reported many years earlier by Treillard & Lwoff (1924) in chironomids from the same source. Very recently, through the kindness of Dr Barthelmes, I have had an opportunity to study tetrahymenae found in chironomids from carp ponds near Berlin: these ciliates also appear to be identical with those I observed in my Parisian material.

Treillard & Lwoff (1924) considered their organism to be just another strain of '*Glaucoma piriformis*', using a specific name now reduced to synonymy with *Tetrahymena pyriformis*. In 1952, I tentatively concluded (Corliss, 1952*c*) that the chironomid parasite should be assigned to the species *T. parasitica* (Penard, 1922) Corliss, 1952, in a category arbitrarily designated subdivision 'a' of that species. However, since that time the genus has doubled in size (six to twelve species), and fresh comparative study of all strains and species which I consider to be congeneric has convinced me that the organism should be recognized as a distinct and separate species, here named *T. chironomi* sp.nov., the thirteenth species assignable to the genus.

It is a pleasure to acknowledge the special interest and valued counsel of several colleagues during the long period of preparation of the present paper: Professor E. Fauré-Fremiet, Collège de France, Paris, in whose laboratory much of this work was carried out; Dr Barbara Walshe Maetz, l'Institut Océanographique, Paris, who collaborated with me in an experimental part of this study; Dr Detlev Barthelmes, Institut für Fischereiwesen der Humboldt-Universität, Berlin, who kindly supplied some preparations of Berlin material; Dr C. A. Hoare, F.R.S., Wellcome Laboratories of Tropical Medicine, London; Dr J. D. Stout, Soil Bureau Experimental Station, Lower Hutt, New Zealand; Dr J. C. Thompson, Jr., Hollins College, Virginia; Dr E. N. Kozloff, Lewis and Clark College, Portland, Oregon; and Dr Ruth McClung Jones, Winthrop College, Rock Hill, South Carolina.

II. MATERIALS AND METHODS OF INVESTIGATION

The midge (chironomid) larvae from which the new species of *Tetrahymena* was isolated were supplied by Monsieur L. Presle, proprietor of the 'Au Pêcheur Moderne', in Paris. They were delivered to the laboratory at the Collège de France in small packets of wet moss, generally 100–200 larvae to a packet. Presle's source remains unknown. Identification of these *vers de vase* as very likely *Chironomus plumosus* was kindly confirmed by Dr Barbara Walshe Maetz who has worked on physiological problems involving the larvae of this and related species of midges for some years.

A brief explanation is necessary here concerning the name I have chosen to use for this nematoceran insect, although full treatment of the legalistic complications surrounding the nomenclatural problem involved is well beyond the scope of the present paper. Meigen (1803), ignoring his own earlier work (Meigen, 1800), erected the genus *Chironomus* with *Tipula plumosa* L. as type, an action long accepted by most entomologists. In recent years some workers have felt that one of Meigen's 1800 names, *Tendipes*, should be revived and substituted for the more familiar, well-established *Chironomus*, strictly on grounds of priority. No one

can deny that *Tendipes* is the earlier name proposed for the group of insects under discussion. Certain of these investigators (e.g. Johannsen & Townes, 1952) have gone a step further and replaced the family name Chironomidae by Tendipedidae. But there are recent indications that the new *International Code of Zoological Nomenclature* will, in the interests of stability, insist upon retention of a family name even if the generic name upon which it originally was based has fallen into synonymy (Bradley, 1957; Hemming, 1953; Mayr, Linsley & Usinger, 1953). With regard to the present problem, then, the family name Chironomidae will be officially retained and the vernacular name 'chironomid' will remain wholly appropriate. Furthermore, it is evident that many British, German, and American writers on the systematics, ecology, and physiology of this group are continuing to favour use of the firmly entrenched generic name *Chironomus* (e.g. see such standard references as Borror & DeLong, 1954; Brues, Melander & Carpenter, 1954; Imms, 1957; Thienemann, 1954; Wigglesworth, 1950). Thus I choose to ally myself with those zoologists and entomologists who feel strongly that the 'Law of Continuity and Universality of Usage' should be invoked in such a case* rather than the 'Law of Priority.'

No attempt was made to maintain cultures of most of the chironomids which were examined for the presence of ciliates. One experiment, designed to determine the length of life of parasitized larvae, however, required preservation of the hosts. This was carried out by keeping the animals in small but properly aerated vessels, set up with the help of Dr Maetz.

Discovery of ciliates in the haemocoele of washed chironomids† was carried out by use of a standard dissecting microscope, with transmitted light. The transparency of the body wall is sufficient to allow detection of the active parasites within the insect (see Figs. 1–3). Heavily infested hosts contained thousands of ciliates; the protozoa were thus readily available in abundance for study in the living condition under the higher powers of a compound microscope or for fixation and subsequent staining in order to obtain permanent slide preparations.

Several free-living strains believed to be conspecific with the forms found in the body cavity of *C. plumosus* were isolated from fresh-water habitats in the vicinity of Paris (Chantilly and Michaud) and subjected to the same methods of study.

The principal cytological staining techniques employed were the Chatton–Lwoff silver impregnation method (as described by Corliss, 1953*b*) and the Feulgen nucleal reaction. Combined with observations on living material, the morphological characteristics revealed by these two techniques made possible an accurate analysis

* Naturally this matter is one to be settled by the International Commission on Zoological Nomenclature, and we all must follow its decision. To my knowledge, no opinion has been published yet on the subject.

† Interestingly enough, in addition to finding two tetrahymenid species within the insect larvae (see footnote p. 112), a third, *T. rostrata* (Kahl, 1926) Corliss, 1952, was isolated from the moss in which the larvae were wrapped! *T. rostrata* is strongly histophagous in its feeding habits (Kahl, 1931; Corliss, 1952*d*; Stout, 1954) and has been isolated from terrestrial annelid and molluscan hosts (e.g. by Stout, 1954; Kozloff, 1957; Kazubski, 1958; and others), but it was never found inside of or feeding upon a living or moribund chironomid during the course of the present investigation.

of features important in the comparative systematics of these and the related tetrahymenid ciliates involved. The value of the infraciliature (essentially the argentophilic basal granules or kinetosomes comprising the subpellicular bases of cilia and compound ciliary organelles), in particular, in comparative studies of ciliates at both the lower and higher taxonomic levels has been stressed elsewhere (Corliss, 1956*a*, 1959).

Several permanent preparations, silver and Feulgen, of ciliates from Berlin specimens of *C. plumosus* also have been studied. Dr Barthelmes kindly sent me these slides with permission to include in the present paper any morphological data resulting from my examination of them.*

Cytological preparations of all species, facultatively parasitic or not, presently included in the genus *Tetrahymena*, as well as several from related genera, were studied first-hand. I am indebted to Dr J. Muspratt, South African Institute for Medical Research, Johannesburg, for supplying me with fixed specimens of '*Lambornella stegomyiae*' from which I was able to make several helpful slides. Unfortunately re-appraisal of early reports of parasitism involving tetrahymenid ciliates had to be limited almost entirely to scrutiny of the published texts and figures available in the literature.

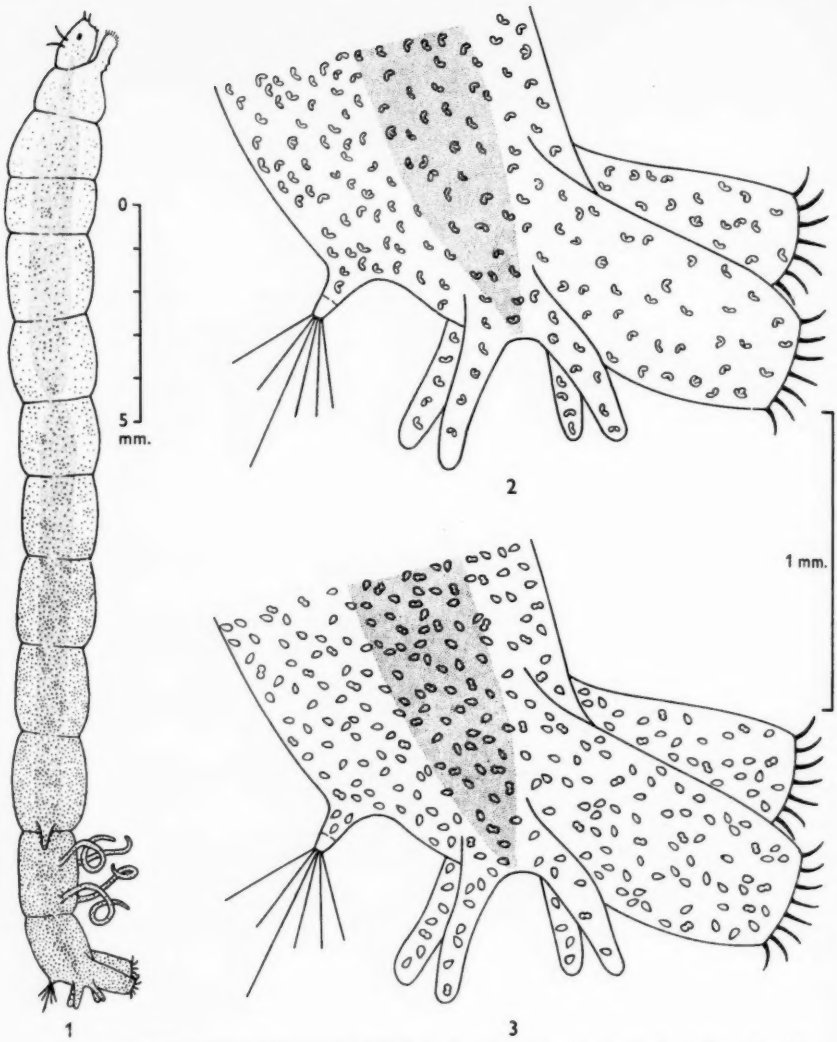
All measurements of protozoan material given in this paper were made by use of a calibrated ocular micrometer. Unless otherwise stated, all data represent means based upon a large number of measured specimens.

III. MORPHOLOGICAL DESCRIPTION OF *TETRAHYMENA CHIRONOMI*

The present section is limited, for the most part, to data obtained by study of the Parisian strains of this ciliate found in chironomid larvae. Lack of knowledge concerning the exact natural source of these commercial 'mud worms' precluded examination of free-living ciliates from the same fresh-water habitats. Other ciliates not isolated from chironomids but considered identical were not investigated extensively enough to be given parallel treatment here. Similarly the limited observations by Treillard & Lwoff (1924) of their strain and by myself of Barthelmes's Berlin material are not considered in detail. But it is important to note that such data serve to confirm, and never contradict, the diagnostic features reported below. Comparison with other species in the genus is reserved, in general, for a subsequent part of the paper (see § V).

Body form and size. The typical shape of *T. chironomi* is pyriform; it is indistinguishable from the type species, *T. pyriformis*, in this characteristic (cf. Figs. 6*a*, *b*; 7*a*, *b*). The size of the organism as generally found in the host or after release into an aqueous medium averages about $40 \times 23 \mu$ with upper limits of length only slightly over 50μ. Free-living strains isolated from other sources averaged a little larger in size. Pre-conjugants, conjugants, and ex-conjugants, however, are

* While this paper was in press, I heard from Dr Barthelmes that his interesting ecological observations on the occurrence of tetrahymenid ciliates in chironomid larvae from certain carp ponds have been accepted for publication in the *Zeitschrift für Fischerei*.



Figs. 1–3. *Chironomus plumosus*, fourth-instar larva, quite heavily infected with the facultative parasite *Tetrahymena chironomi*. Fig. 1. Outline drawing of entire larva, with digestive tract shown by light stippling, as seen with transmitted light. Note the thousands of ciliates, depicted as mere specks at this magnification, throughout the body cavity, with heaviest concentrations in the posterior portions of the host's haemocoele. The parasites surround, but are not within, the intestine. Fig. 2. Enlarged, semi-schematic drawing of posterior end. A picture frequently seen in long-infected larvae soon to die: all the ciliates, now noticeably smaller in size (cf. Fig. 3), are synchronously in conjugation within the crowded haemocoele. A few trios may be detected among the conjugating pairs. The type of conjugation undergone by *T. chironomi* in (or even when released from) *C. plumosus* is a lethal, selfing kind. Fig. 3. Same setting, but an earlier stage of the infection. Note that here, too, the tetrahymenae, a number of which are actively undergoing fission, are to be found in great numbers everywhere, even in the anal gills and the pre-anal papillae. In all of these figures the ciliates are shown as if in a single plane; the total number actually present would, of course, be much greater.

[I am indebted to Miss Alice Boatright for aid in final preparation of Figs. 1–7.]

noticeably smaller* and leaner, averaging about $26 \times 16\mu$, and seldom reaching a length over 35μ.

Minor features related to body form and size, such as the extreme pliability of the pellicle and the occasional occurrence of anomalous shapes, are common among all species of the genus (for example, see Corliss, 1953*a*) and do not warrant redescription here. No cystic stages have been noted in the life cycle; also no striking dimorphism, no cannibalism, and no occurrence of homopolar doublets. The appearance of trios amongst conjugating pairs is mentioned below in a special consideration of the sexuality exhibited by this species (§ IV).

Cilia and ciliary meridians. General features of the somatic ciliature are typical of the genus. No longer caudal cilium was observed and no polar basal granule-complex was found at the posterior pole of silver-impregnated specimens.

The number of meridional rows of cilia ranges from 24 to 28, inclusive, with 24–26 most commonly found. The same range exists in the non-parasitic strains studied; in Berlin material I have occasionally detected individual ciliates possessing as few as 23 rows of somatic ciliature. Two ciliary meridians are terminated anteriorly at the posterior margin of the buccal overture. The right-hand one of these two rows is the stomatogenous or number one meridian.

Concerning the detailed nature of the meridians, as revealed in silver preparations, it is interesting to note that essentially no secondary meridians are present. In several of the other species of *Tetrahymena* such structures do occur, generally recognizable as non-granular, argentophilic lines, more or less continuous, located approximately midway between the primary (true kinetosome-bearing) meridians (cf. Figs. 6*a* and 7*a*).

Characteristics of the buccal apparatus. The tetrahymenal equipment of compound ciliary organelles, the tripartite adoral zone of membranelles on the left plus the single undulating membrane on the right, is identical in all respects with that now well-known for the type species, *T. pyriformis* (see Figs. 6*a* and 7*a*, and Corliss, 1953*a*, and references therein). Several inexplicable instances of forms with only vestigial oral equipment were encountered. In such cases hundreds of the ciliates were mouthless, but seemed normal in other respects. These organisms even underwent conjugation, outside the host, some hours after they were first discovered. Ex-conjugants apparently were always astomatous, even in the usual cases in which the preconjugants were clearly in possession of the normal buccal apparatus. This is not startling in view of the fact that the conjugation inevitably was of a selfing nature and all the organisms were destined to die (see § IV, below).

In dividing forms the buccal anlagen for the posterior daughter, the opisthe, arise, as expected, from a field of granules derived from the stomatogenous meridian number one. As noted above, no two-mouthed forms, homopolar doublets, have been seen in this species.

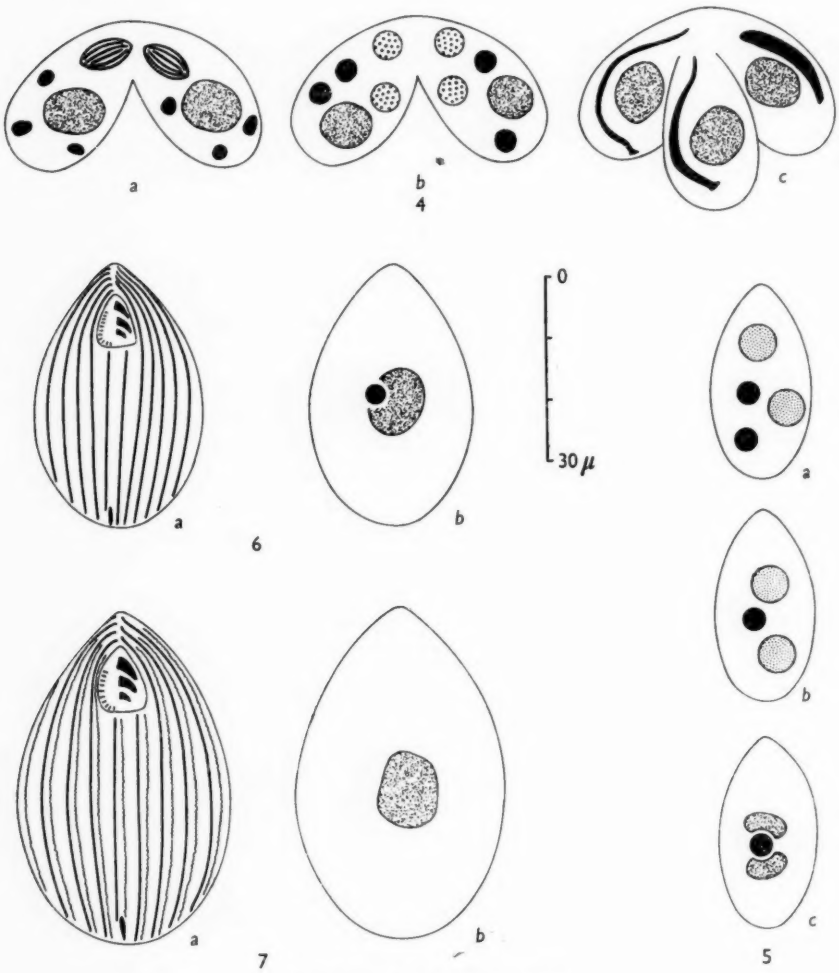
Cytoproct and contractile vacuole pores. The cell anus or cytoproct is located terminally in meridian number one, as is typical of all species in this and closely

* But, according to Treillard & Lwoff (1924), both the conjugants and the non-conjugants observed by them were all of the same size, averaging $40 \times 28\mu$.

related hymenostome genera. It is most conveniently detected in silver-impregnation preparations.

The contractile vacuole pores, two to four, most often three, in number, are located in the vicinity of the posterior ends of the correspondingly shortened ciliary meridians on the right side of the body. The meridians involved are numbers 6–9, generally 6–8. These minute, frequently overlooked but taxonomically important argentophilic structures occur in a general position characteristic for the genus *Tetrahymena*.

Nuclear characteristics. The single, chunky, ovoid to irregularly spherical macronucleus is centrally located in the body and generally measures less than 12μ in its longer axis. The single, spherical micronucleus is located nearby, often in a cleft



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or notch of the macronucleus (see Fig. 6*b*). In the *T. chironomi* specimens from infected hosts the micronucleus is relatively large for species of this genus, its diameter ranging from 3·0 to 3·7 μ. In strains of the species isolated from nature as free-living ciliates not in direct association with chironomids, the few micronuclei studied ranged from 2 to 3 μ in diameter. Whether this represents a permanent, natural racial difference, or a direct temporary effect of the host on the ciliate, or an indication of the ensuing occurrence of conjugation cannot be determined at the present time.

Macronuclear chromatin extrusion has been seen in Feulgen preparations of dividing forms. This inexplicable phenomenon is quite widespread among hymenostome ciliates. The possible significance of its occurrence in species of *Tetrahymena* is currently under investigation (Dysart, 1959).

IV. SEXUALITY IN *TETRAHYMENA CHIRONOMI*

Occurrence of conjugation. The most striking feature of the organism studied by Treillard & Lwoff (1924), according to those workers, was its exhibition of conjugation. In five of the thirteen parasitized larval chironomids examined by them conjugation of the ciliates was in progress. In the much more extensive survey which I made in Paris, some 27 years later, conjugation in the ciliate populations

Figs. 4–7. Semi-schematic drawings, all to the same scale, of the two ciliates, *Tetrahymena chironomi* and an amicronucleate *T. pyriformis*, found as facultative parasites in the body cavity of larval *Chironomus plumosus*. All nuclear structures as revealed by the Feulgen nucleal reaction. Fig. 4*a–c*. Selected stages in the conjugation exhibited by *T. chironomi*. Fig. 4*a*. Post-meiotic division (a meta-anaphase stage) of the micronucleus which will form the two haploid pronuclei in each conjugant. Note the large, original macronuclei and the disintegrating relic products of meioses I and II. Fig. 4*b*. Results of the second reorganizational (post-synkaryonal) divisions: two micronuclear and two macronuclear anlagen, the macronuclear bodies the more lightly stained. The 'old' macronuclei still persist, although now rapidly becoming reduced in size. Fig. 4*c*. An example of a trio attempting conjugation. The elongate structures are the micronuclei in early prophase of meiosis I. These curious crescent-forms are typical of all species of *Tetrahymena* studied. The stages depicted are progressively more developed (older) from right to left. Fig. 5*a–c*. Exconjugants, showing three typical stages in the nuclear picture. Fig. 5*a*. Two presumptive nuclei of each kind: a stage briefly seen. Fig. 5*b*. One of the micronuclei suddenly has been totally resorbed. A long-lasting stage. Fig. 5*c*. Final stage seen. The two macronuclear anlagen, now looking quite similar to normal vegetative macronuclei of the non-conjugating ciliate, typically are cupped about the single, large, spherical micronucleus. In *T. chironomi* the organisms all die in this stage. Fig. 6*a*, *b*. *T. chironomi*, ventral view; the non-conjugating ciliate is considerably larger than the selfing conjugants. Fig. 6*a*. Silver-impregnated specimen with all silverline structures shown diagrammatically, particularly the argentophilic basal granules comprising the (primary) ciliary meridians here shown as solid black lines. Cf. Fig. 7*a*, and note three taxonomically important differences: body size, number of meridians, nature of meridians (no secondaries between the primaries in *T. chironomi*). Fig. 6*b*. Normal nuclear picture, as seen in Feulgen preparations. Cf. Fig. 7*b*, for a further distinctive difference between the two ciliates sometimes found in the same host chironomid larva. Fig. 7*a*, *b*. *T. pyriformis*, amicronucleate strain from chironomids, ventral view. Fig. 7*a*. Silver-impregnated specimen; cf. Fig. 6*a*, above. Fig. 7*b*. Note total absence of micronuclear material, as seen in Feulgen preparations.

once again was noted with high frequency. Very recently Dr Barthelmes has written me of his own discovery of this phenomenon among the ciliates in the body cavity of Berlin chironomids. In all cases essentially 100 % of the organisms in or from a given host specimen have been engaged in this form of sexuality at the same time.

Certain aspects surrounding the occurrence of conjugation in facultatively parasitic members of *T. chironomi*, in which the intimacy of the host–parasite relationship is concerned, are reserved for later consideration (§ VI, below). But several descriptive observations should be reported here. Conjugation can occur in the body cavity of larvae of any size, but only when the infection is heavy, i.e. tens of thousands of ciliates per host (see Fig. 2). Pairing of the protozoa regularly takes place about 2 days before the death of the host. Deliberate manipulation of the chironomid's laboratory environment to hasten its death causes the onset of the ciliate conjugation to commence equivalently earlier. If the parasites are released from the host, by breaking open the chironomid's body with dissecting tools, from one to many hours before conjugation has commenced, then the ciliates, at the appropriate time, engage fully in their sexual activity in the culture dish quite free from the host's body. Incidentally, in several cases I have observed trios among the pairs of conjugants. Cytological examination has shown that meiotic phenomena are undergone in all three organisms but the exact relationship of the three has been indeterminable.

Fate of the exconjugants. Following conjugation, the exconjugants are easily seen as small, actively moving forms present in abundance in the haemocoele of the host. If the moribund, and eventually dead, body of the chironomid larva is left undisturbed the exconjugants manage to escape in due time. This is permitted by decomposition or bursting of the insect's gut or by the natural occurrence of breaks in the body wall. Practically all, if not all, exconjugants, regardless of their source, die: establishment of viable cultures, either bacterized in a variety of media or axenic in proteose-peptone, never has been successful, although attempted repeatedly.

One particular set of observations may be cited as an example of the timing in the occurrence of conjugation and its subsequent disastrous effect on the life of the ciliates involved. Careful examination of a fairly heavily infected fourth-instar larva showed that no conjugation, only fission, was evident in the protozoan population. Exactly 48 hr. later the host was still actively alive and no conjugation of the ciliates was seen. At this time the chironomid was sacrificed and the released protozoa were placed in several depression slides. Conjugation was detected 18 hr. later, having swept simultaneously through the cultures in epidemic proportions. Few single individuals were to be found among the pairs and cytological staining revealed that the micronuclear activities were predominantly in stages of the first meiotic division. But some 8 hr. after this only exconjugants were to be seen: staining showed them to possess two micronuclei and two macronuclear anlagen. Subsequent observations were made after additional periods of 15, 21 and 46 hr. In the first two of these, typical, fast-moving exconjugants still were in great abundance, and the nuclear situation had reached the relatively stable

situation of a single micronucleus and a pair of macronuclear anlagen cupped about it (see Fig. 5*c*). But in the last observation, exactly 72 hr. after release of the tetrahymenae from the host, every one of the exconjugants had succumbed.

Selfing nature of the conjugation. The most curious feature about the conjugation habitually exhibited by *T. chironomi* from midge larvae is its apparent *selfing* nature. Although it was impossible to carry out any genetic studies on the problem, the circumstantial evidence that it is truly a selfing type of conjugation is strong. These points may be considered to support the conclusion: when all the ciliates parasitic in a given host belong to the species *T. chironomi*,* they all undergo conjugation; this happens repeatedly and independently in many separate individuals of the host animal; mortality is extremely high, a well-known feature of selfing. It is thus unlikely that the proper complementary mating types of 'normal' conjugation are randomly entering the chironomid in approximately equal numbers; indeed, Treillard & Lwoff (1924) believe that most larvae are initially infected by a single ciliate (see § VI, however).

Selfing types of conjugation have long been seen but have been little studied and even less understood. Modern investigations involving strains only of *T. pyriformis* of the genus *Tetrahymena* have been and are being carried out principally by Nanney and co-workers (for example, see Allen & Nanney, 1958; Nanney, 1953, 1954, 1956; Nanney & Caughey, 1953, Nanney, Caughey & Tefankjan, 1955). Some sort of macronuclear instability with regard to mating type genes is postulated to explain, at least in part, the natural occurrence of selfing in *T. pyriformis*. In the case of *T. chironomi* some factor or alteration in the chemico-physical make-up of the parasitized chironomid's body fluids is very likely involved in triggering the onset of conjugation in the ciliate population. By application of modern techniques of genetics and biochemistry the important problem could be attacked most profitably; until that is accomplished, further speculation is of little value.

It is very interesting to note, incidentally, that Warren (1932), in studying *T. limacis* from slugs, also reported epidemics of conjugation in cultures of ciliates established after their release from the host. And the exconjugants all died. Interestingly enough, Grassé & de Boissezon (1929) mentioned in a footnote in their paper on a mosquito parasite (see § V for consideration of its systematic status) that some forms showed two macronuclei and one micronucleus: surely these were exconjugants (see my Fig. 5*b*).

It should be stressed, however, that 'host fluids' are not indispensable for selfing in all *Tetrahymena* strains in which this type of conjugation has been seen. The *T. pyriformis* cultures studied by Nanney and co-workers were not parasites; Horn (1951, unpublished) once isolated a selfing strain of free-living *T. pyriformis* (in fact, she was the first to discover sexuality in this species); and I have observed (unpublished notes) selfing in the type species, *T. pyriformis*, and in bacterized cultures of *T. limacis*, strain D.r.IV, grown from organisms originally received from Dr E. N. Kozloff. In the last-mentioned case, the strain's natural source was

* In some cases *T. pyriformis* is simultaneously present; see footnote p. 112, and §§ V and VI.

from a slug, but subsequently it had been living in laboratory cultures for hundreds of generations free from any influence of its former host. Kozloff (1956*b*, *c*) also has reported selfing in many laboratory strains of *T. limacis*.

Cytological aspects. The cytological picture of conjugation in *T. chironomi* follows what may be called the usual *Tetrahymena* pattern and thus it need not be described in detail here. Actually Treillard & Lwoff's (1924) observations, generally overlooked by workers in the field of protozoan cytogenetics, are quite accurate, although limited in scope. Still earlier, Maupas (1888) described conjugation in *T. patula* exceedingly well for the times in which he worked. Now the excellent descriptions of Elliott & Hayes (1953), Nanney (1953), and Ray (1956), on *T. pyriformis*, are available, as well as a preliminary note on the cytological picture of autogamy in *T. rostrata* (Corliss, 1952*d*).

The most striking stage noted by all workers in all species is the 'crescent' stage occurring in the prophase of the first meiotic division of the micronucleus (see Fig. 4*c*). It is in the very next stage that the tetrads are so arranged as to allow an opportunity to count the chromosomes. Delicate cytological procedures are required, however, to be able to ascertain the number with complete accuracy. To date only Ray (1956) has been eminently successful in this respect; from his pioneering work we know that $2N = 10$ in the sexual strains of *T. pyriformis*. There are estimated to be several times as many chromosomes in *T. rostrata* (Corliss, 1956*b*), where they are small and crowded together, somewhat reminiscent of the chromosomal constitution reported for certain strains of *Paramecium aurelia* (Dippell, 1954).

Characteristic of the exconjugants are the two macronuclear anlagen cupped around the single, prominent micronucleus (see Fig. 5*c*).

V. SYSTEMATIC STATUS OF *TETRAHYMENA* SPECIES, PARTICULARLY THOSE EXHIBITING FACULTATIVE PARASITISM

General considerations. In the short period of some 10–12 years during which I have been working actively on the systematics of tetrahymenid ciliates the problem of where to draw specific lines within the genus *Tetrahymena* has increased greatly in complexity.* The two oldest species, *T. pyriformis*, the type species, and *T. patula*, representing the taxonomic extremes of the genus from several considerations, are generally easily distinguishable. As additional species have been described, the compactness of the group has become more apparent; at the same time the boundaries of the entire genus have had to be expanded somewhat; e.g. to accommodate forms with a caudal cilium (Holz & Corliss, 1956). Whereas the several species known in 1952 could be separated neatly solely on the basis of clear-cut differences in mean or modal numbers of ciliary meridians (Corliss, 1952*c*; 1953*a*), subsequently two complications have arisen. A number of species are known to possess similar numbers of meridians and thus are distinguishable only on the basis of other single characteristics or, more often, of combinations of

* On the other hand, there is no particular problem, today, in separating ciliates within the family Tetrahymenidae at the *generic* level (Corliss, 1952*c*, *f*, 1953*c*, 1954*b*).

characteristics. And a lack of stability of numbers of ciliary rows *within* a given species has been shown to exist, under the influence of varying environmental conditions, in certain forms (Kozloff, 1956*b*, *c*, 1957).

A major complication in the case of the several 'new' forms first described from parasitic material, as will become evident below, is that often the accounts are quite inadequate for the comparative purposes so essential to the over-all problem. Numbers of rows of cilia, for example, were seldom given, or were mentioned in passing in the text and then shown quite differently in accompanying figures. Unfortunately, the ciliates involved are small and relatively undifferentiated, and the earlier workers cannot be blamed for failing to employ such ideal techniques as silver impregnation which had not yet been introduced into protozoology.

Further detailed discussion of such problems must be reserved for lengthier treatment in a forthcoming monographic review and revision of the genus *Tetrahymena** on which I have been working for several years. That the particular matters mentioned above are very pertinent to the subject of the present paper, however, is obvious. At least brief comparison of *T. chironomi* with the other twelve members of the genus is required here. Possible disposition of even the more poorly known forms in the literature on accidental and facultative parasitism also is made, with deliberate omission of details. Sometimes the taxonomic decisions made are inevitably highly subjective ones; only rediscoveries and precise redescriptions (which may never come to pass) can clear up satisfactorily the problems involved in the most suitable allocation of certain strains and species.

In Table 1 (pp. 130–7) may be found, along with other information, a summary of my taxonomic conclusions regarding all the tetrahymenid ciliates described from other organisms to date.

Tetrahymena chironomi *and* T. pyriformis. That *T. chironomi* is congeneric with the type species, *T. pyriformis* (Ehrenberg, 1830) Lwoff, 1947, hardly needs to be stated. Indeed, it is most closely related to this organism, as may be noted from the data given in the morphological description above (§ III; and cf. Figs. 6*a*, *b*; 7*a*, *b*); and it may be recalled that Treillard & Lwoff (1924) considered the two ciliates to be identical. Both, of course, possess the strong *generic* characters of the tetrahymenal buccal apparatus, general arrangement of ciliary meridians, location of cytoproct and contractile vacuole pores, etc. In addition, the shape of the body is identical and the life cycle appears to be essentially monomorphic in both cases. *T. chironomi* is smaller in size, on the average, but there is a large area of overlap in the ranges: the mean for trophonts of *T. chironomi* is $40 \times 23\mu$, with maximum lengths under 60μ; the mean for *T. pyriformis* is about $50 \times 30\mu$, with maximum lengths approaching 100μ.

The number of ciliary meridians differs significantly. In the dozens of strains of *T. pyriformis* which I now have studied, including both micronucleate and amicro-

* This generic name is used advisedly in spite of the fact, which I have pointed out elsewhere (Corliss, 1953*a*, 1954*a*; Corliss & Dougherty, 1955), that legally it is antedated by several other names. A petition, containing all the necessary historical and nomenclatural details, has been formulated in which the International Commission on Zoological Nomenclature is asked to employ their plenary powers to declare *Tetrahymena* Furgason, 1940, a *nomen conservatum* (Corliss & Dougherty, 1961).

nucleate races, the full range is from 15 to 23, with both extremes seen very rarely and only in scattered individual specimens from strains with otherwise 'normal' ranges, such as 17–21. In *T. chironomi* taken from Parisian larvae of *C. plumosus* I have found the number to be 24–27, with the upper extreme infrequently encountered. In certain of the same larvae amicronucleate ciliates identifiable as *T. pyriformis* also were discovered (see § VI, below); it is interesting to note that these forms showed only 18–20 ciliary meridians.

There are several minor differences associated with some of the silverline structures. The number of contractile vacuole pores often is greater by one in *T. chironomi* and these taxonomically important landmarks are located in ciliary meridians of slightly higher number. The lack of secondary meridians further distinguishes *T. chironomi* from many strains, particularly the amicronucleate ones, of *T. pyriformis*. The micronucleus typically is much more prominent, sometimes double in diameter, in the species being described as new in this paper.

Tetrahymena chironomi *and* T. parasitica. There are several difficult problems involved here. Up to the present time the ciliate from chironomids has been considered a member of the species *T. parasitica* (Penard, 1922) Corliss, 1952, based upon early decisions of mine (Corliss, 1952*c*, 1953*a*). In 1952 the entire genus was only half its present size and it seemed advisable to place facultatively parasitic forms in the species described as *Glaucoma parasiticum* by Penard (1922) unless they were positively identifiable as belonging to other, distinct species. It could be argued that proposing new names for newly found forms might give rise to considerable confusion in the subsequent taxonomic literature if some of these names were later obliged to fall as synonyms of the older specific name '*parasitica*'.

But one of the problems, still unresolvable, concerned the exact identity of Penard's organism. It was found in the gills of *Gammarus pulex* taken from Lake Geneva and considered by the Swiss investigator to be somewhat different from '*Glaucoma pyriformis*'. It is not difficult to reinterpret his rather incomplete description in the light of modern knowledge, to the extent of recognizing his '*G. parasiticum*' as congeneric with our *T. pyriformis*. But exactly what are its own specific characteristics? How many ciliary meridians does it have? Penard remarks only that its ciliation is more dense.

Thus I have come to agree with Kozloff's (1956*b*, p. 21) judicious suggestion, 'It may be preferable...not to refer any ciliates to Penard's species until tetrahymenas from *Gammarus pulex* have been studied more carefully.' And it should probably be Lake Geneva gammarids, as a further safety precaution.

So *T. parasitica* occupies an uncertain position in the genus, and *T. chironomi* runs the risk of some day being found to be identical with Penard's earlier described species. Unless or until that happens, it seems advisable to keep the forms separate, particularly since future investigators can always be certain of the identity of *T. chironomi*, a species which may become of considerable importance in experimental work.

The problem of the possible relationship between *T. parasitica* and *T. limacis*, just as insoluble, is considered next.

Tetrahymena chironomi *and* T. limacis. Involved here is my earlier decision

(Corliss, 1952*c*, 1953*a*) to consider two poorly described ciliates as identical: Warren's (1932) *Paraglaucoma limacis* and Penard's *Glaucoma parasiticum*, uniting them both, along with my chironomid parasite, as members of *T. parasitica*. In particular, there seemed to be good reasons for considering the *T. chironomi* of the present paper as identical with Warren's form. That investigator's organism was discovered as a facultative parasite in the common grey garden slug, *Deroceras reticulatum*, underwent a selfing type of conjugation, was surely a *Tetrahymena*, and supposedly possessed about 26 meridians (so stated in Warren's text but not supported by his drawings).

The problem was complicated by an early paper of Kozloff (1946) in which he described with precision a ciliate from the same slug species. He concluded that he had a strain of Warren's species and considered the correct name to be *T. limacis* (Warren, 1932) Kozloff, 1946. But his strain possessed about 32–37 ciliary meridians which I believed excluded it from *T. parasitica* (as represented by Warren's slug and my chironomid strains with their 24–26 rows of cilia). On the other hand, that number seemed to make Kozloff's organism conspecific with another species, *T. faurei* Corliss, 1952, isolated from an embryonic trout (see below). Subsequently Kozloff (1956*b*, *c*) has described more slug, and also snail, parasites which he assigns to *T. limacis*. In these not only has the number of ciliary meridians varied in nature, in different strains, from 26 to 40, but those showing numbers in the upper range in the parasitic phase have dropped to the lower end of the range in laboratory cultures of a free-living phase.

Thus I am prepared to revise my own earlier provisional conclusions. I accept Kozloff's decision to lump Warren's parasite and the parasites of his mentioned above to form the single species *T. limacis*. I realize that the *limacis*-group of tetrahymenae, if not others, are able to show a variation in number of ciliary meridians which renders it inadvisable to put full weight on this feature alone as a differential characteristic of prime value. In the case of *Tetrahymena* species exhibiting facultative parasitism other diagnostic factors, including host preference, perhaps have been too much neglected in the past.

T. chironomi, then, may be considered to differ from *T. limacis sensu lato* in several features: it has been found in association with quite a different host; its body shape is different (*T. limacis* is apiculate in its parasitic phase); it is regularly of smaller size (*T. limacis* averages about 10μ longer); it appears to show a more stable and conservative range in its number (typically smaller) of meridians, with no variation inside or outside of the host; it possesses a micronucleus of larger diameter.

Tetrahymena chironomi *and* T. stegomyiae *comb.nov.* The taxonomic status of the ciliate found in larval *Aëdes* (*Stegomyia*) *scutellaris* from the Federation of Malaya by Lamborn (1921) and studied by Keilin (1921), who named it *Lambornella stegomyiae*, has long puzzled protozoologists. The fact that Keilin was obliged to examine material limited in amount and fixed only in dilute formaldehyde and that the degree of relationship, if any, between certain 'cuticular cysts' and the parasitic ciliate could not be ascertained from the preserved material made judgment by subsequent reviewers all the more difficult. In the meantime, primarily

through the works and opinions of Lwoff (1932) and Wenyon (1926), it was becoming common practice to consider '*Glaucoma pyriformis*' as the only holotrich ciliate capable of living under the conditions imposed by such facultative parasitism. Muspratt (1945, 1947), by his own discovery of and experimentation with a ciliate parasitic in certain tree-hole breeding culicine mosquito larvae, really solved the mystery, we can say in retrospect; unfortunately, he chose to consider the protozoon to be '*G. pyriformis*' and his papers lack very necessary data concerning the morphology of the ciliate. In May and June 1958, Dr Muspratt kindly sent me some newly collected material, fixed in de Fano's fluid, from larval *Aëdes* (*Stegomyia*) *metallicus* and *A.* (*S.*) *aegypti*. Study of preparations of this allows me to conclude that his and Keilin's organisms are the same and are members of a distinct species, *T. stegomyiae* (Keilin, 1921) comb.nov.

It is well beyond the scope of the present paper to discuss the morphology of *T. stegomyiae* in detail. In many ways it resembles the type species, *T. pyriformis*, and other members of the genus. Its pear-shaped body generally is larger than that of *T. pyriformis*, frequently being 60–100μ, and its ciliation is denser: there are between 25 and 30 rows of cilia. The tetrahymenid buccal apparatus is typical of the genus. The micronucleus is prominent, spherical, and has a diameter of 2·5–3·0μ or greater. The cysts, which are readily formed on the outside of uninfected *Aëdes* mosquito larvae introduced into containers rich with the ciliates, are about 35μ in diameter and contain nuclei comparable in size, shape, and composition to those present in the active stages of the protozoon. A curious, highly refractile structure about 1·5μ in diameter, apparently a contractile vacuole pore, is present on the upper surface of the cyst. Muspratt is convinced that invasion of the haemocoele is effected from this encysted stage by direct penetration of the ciliate through the cuticular layers of the larval mosquito's body; Keilin had postulated that the mode of entry was via the mouth, then through the intestinal wall. Both investigators considered the organism, which kills the mosquitoes which it invades, to be a true, rather than facultative, parasite. Sexuality has not been reported. Although more information concerning the bionomics of this species would be helpful, its individuality would appear to be sufficiently established by the data given above.*

T. chironomi differs from *T. stegomyiae* in several significant respects: it is smaller, has fewer ciliary meridians, does not form cysts, and is found associated with a

* In 1954 and 1955, Mr J. A. Reid, Institute for Medical Research, Kuala Lumpur, Federation of Malaya, sent me some very interesting fixed material from the abdominal cavity of *adult* mosquitoes belonging to the species *Armigeres* (*Leicesteria*) *dolichocephalus*. Unfortunately difficulty was experienced in making good silver-impregnation preparations from the material: e.g. either the ciliates involved were mouthless or their buccal structures were not visible in the few specimens in which other silverline structures are revealed. In a pyriform-shaped organism some 50–60μ in length about 50 meridians are present; but in huge, nearly spherical specimens from the same material about 200 meridians are detectable! It is not known if the two kinds of protozoa represent stages in the life history of a single species. This case must remain enigmatic until new samples are available for study. It is believed worthwhile to mention it here, although this is not to imply that I consider Reid's Malayan protozoa to be identical with Lamborn's ciliates found in a species of larval *Aëdes* collected from the same part of the world; in fact, Reid's very curious organisms may be quite unrelated to the species presently comprising the genus *Tetrahymena*.

different host. Since *T. chironomi* does not form cysts on chironomid larvae, its mode of entry into its host must be quite distinct from the method apparently exhibited by *T. stegomyiae*.

Tetrahymena chironomi *and* T. faurei. The only strain of *T. faurei* Corliss, 1952, ever studied was one isolated from the cerebrospinal fluid and tail musculature of an injured embryonic trout (Corliss, 1953*a*), and it is now extinct. Kozloff's (1946) strain of *T. limacis* is no longer considered to be a member of this species, as discussed above. In light of our present knowledge, it would be highly desirable, of course, to have more information concerning the bionomics of *T. faurei*, surely only an accidental parasite of the single laboratory-raised trout specimen in which it was found. The species must remain in an uncertain status, similar to that proposed above for *T. parasitica*, until rediscovery and further study become possible.

Nevertheless, it is unlikely that *T. chironomi* will be found to be identical with *T. faurei*. This conclusion is supported by differences in hosts; body size and shape differences (*T. faurei* is larger and possesses an attenuated anterior end in one stage); differences in number of ciliary meridians (about ten more, on the average, in the larger species); and minor dissimilarities in certain features of the silverline system.

Tetrahymena chironomi *and* T. glaucomaeformis. Found only as a free-living form, von Gelei's '*Colpidium glaucomaeforme*' has enjoyed a rather uncertain taxonomic status since it was first described by von Gelei (1935). Kahl (1935) considered it as probably identical with his own *Saprophilus oviformis*. Furgason (1940) transferred it to the genus *Tetrahymena* as a distinct species, *T. glaucomaeformia*; later Kozloff (1946) properly emended its name to read *T. glaucomaeformis*. Mugard (1949), in my opinion, really treated this species under the name of '*Paraglaucoma rostrata*'. In a note reviewing the composition of the genus in 1952, I suggested reduction of von Gelei's organism to a strain level within a temporary subdivision 'd' of *T. pyriformis*, although I soon expressed uncertainty over this disposition (Corliss, 1952*c*, 1953*a*). Recently I have considered Furgason's action as a completely justifiable one (Holz & Corliss, 1956), although *T. glaucomaeformis* (von Gelei, 1935) Furgason, 1940, certainly could be restudied profitably.

T. chironomi possesses a number of similar characteristics. But the remarkable consistency in the number of ciliary meridians, a range of only 22–23 with a distinct mode of 23, shown by *T. glaucomaeformis* quite clearly separates the two species. Also the latter species has a smaller micronucleus and shows some differences in body shape and size and in details of the silverline system. The body of *T. glaucomaeformis* becomes noticeably much darker in coloration when fed in the laboratory on animal tissue.

Tetrahymena chironomi *and* T. patula, T. vorax, *and* T. paravorax. Three species in the genus possess in common and are characterized, in part, by an ability to undergo a pronounced dimorphism, microstome ⇌ macrostome, during their life cycle. This striking phenomenon has been described recently in some detail (Corliss, 1953*a*) for the two species *T. patula* (Ehrenberg, 1830*) Corliss, 1951, and

* Formerly 'O. F. Müller, 1786' was used here instead of 'Ehrenberg, 1830'. See Corliss & Dougherty (1961) for discussion of the complicated but purely *nomenclatural* problem involved in making this substitution.

T. vorax (Kidder, Lilly & Claff, 1940) Kidder, 1941, in which it has been known for some time; but only a preliminary communication (Corliss, 1957) exists in the case of *T. paravorax* Corliss, 1957. *T. chironomi* is distinctly set apart from this trio by its own inability to show such dimorphism.

There are also other important differences including the following: none of these three species has been found as a facultative parasite; their body sizes are considerably larger; *T. paravorax* possesses a distinct caudal cilium; *T. vorax* exhibits strong secondary meridians in its silverline picture; *T. patula* is able to reproduce in an encysted state. The number of ciliary meridians in *T. vorax* and *T. paravorax* overlaps the range shown by *T. chironomi*, but *T. patula* shows a considerably larger number (32–45).

Tetrahymena chironomi *and* T. rostrata, T. corlissi, *and* T. setifera. Four species in the genus are known to possess a distinctive caudal cilium extending out from the posterior pole of the organism. In silver preparations this specialized cilium is represented by a unique polar basal granule-complex (PBG-complex) forming part of the argentophilic infraciliature. The species, the first two of which have been found exhibiting facultative parasitism (§ VI, below), are as follows: *T. rostrata* (Kahl, 1926) Corliss, 1952; *T. corlissi* Thompson, 1955; *T. setifera* Holz & Corliss, 1956; and *T. paravorax* Corliss, 1957. The last-mentioned already has been considered in preceding paragraphs.

In addition to the fact that *T. chironomi* is separated from these forms by its own lack of an elongate caudal cilium, several other differences may be mentioned briefly. *T. rostrata*, which has been found as both an edaphic and parasitic species, has more meridians, an ovoid micronucleus, and typically shows a different body shape and size; also it is able to produce both resting and reproductive cysts (Kahl, 1926; Corliss, 1952*c*, *e*; Stout, 1954; Kozloff, 1957; Kazubski, 1958). The sexual phenomenon of autogamy has been discovered to occur with some regularity in the resting cysts (Corliss, 1952*b*, 1956).

T. corlissi has a slightly larger number of meridians (25–31), shows difference in body shape, and produces reproductive, but no resting cysts. This species has been found as a facultative parasite of lower vertebrates (Burns *et al.* 1955; Nance, 1954; Thompson, 1955*a*; Speidel, 1953, 1954, 1958*a*), but also very recently has been recovered from dry moss (McArdle, 1959). Its histophagous proclivities have been used to advantage, incidentally, in isolation of earthworm setae: see Harman & Corliss (1956). *T. setifera* has 22–26 rows of cilia and a body form and size very similar to that of *T. chironomi*. However, it has been found only in free-living environments, has shown no sexuality to date, and, of course, possesses the distinctive feature of the caudal cilium and its prominent infraciliary counterpart, the PBG-complex.

Tetrahymena chironomi *and other forms found as facultative parasites.* Seven of the twelve species of *Tetrahymena* considered above contain strains found in close association with various animals serving as hosts. The systematic status of these species has been discussed above, and aspects of the host–parasite relationship involved are treated below (§ VI). But the taxonomic affinities of *T. chironomi* with still other often poorly known *Tetrahymena*-like ciliates discovered as faculta-

tive parasites remain for consideration here. The major difficulty in drawing conclusions in this area stems, usually, from the dearth of usable data concerning the organisms involved in these little-known cases. As Lwoff (1932, p. 56) long ago pointed out, in his own brief discussion of the problem of proper identification of ciliates associated as parasites with other animals, '. . .il est préférable de se livrer à un sérieux examen de l'infusoire vivant, avec le contrôle, actuellement indispensable, de l'étude de la ciliature après imprégnation à l'argent'.

In the early days of the discovery of new instances of ciliates acting temporarily as parasites it was fairly common practice to name not only the species but even the genus as new. These will serve as examples: *Lambornella stegomyiae*, *Glaucoma parasiticum*, *Balantidium knowlesii*, *Turchiniella culicis*, *Leptoglena knowlesii*, *Paraglaucoma limacis*, *Glaucoma paedophthora*. Then the pendulum swung to the other extreme, and it apparently became fashionable to consider all such incidents as very likely involving but a single species, namely '*G. pyriformis*' (e.g. see remarks by Codreanu, 1930; Lwoff, 1932; Wenyon, 1926; and, to some extent, Kirby, 1941, and Mugard, 1949). This point of view probably represented an improvement, at least taxonomically speaking, although a few very serious errors were made by some workers in suggesting that certain perfectly good *Colpoda* and *Ophryoglena* species, forms belonging to distinctly separate families, for example, were identical or even congeneric with *Tetrahymena pyriformis*.

Critical re-appraisal of the limited data usually provided in the older literature has made possible some fairly reasonable asumptions concerning the taxonomic affinities of certain of the forms briefly described as parasites, particularly with the aid of our present-day knowledge in the field. In other cases exact identification is absolutely impossible. In such instances a subjective decision has been made here: either the ciliate is considered a separate species, if the original description at all warrants it, with the hope that it will be rediscovered and more properly described at a future time; or it is considered as probably or possibly identical with one or another of the known species, as long as the original limited data do not seriously contradict such an allocation. Examples of the first case would include Penard's *Glaucoma parasiticum* (= *Tetrahymena parasitica*), Warren's *Paraglaucoma limacis* (= *T. limacis*) and Keilin's *Lambornella stegomyiae* (= *T. stegomyiae*). As discussed earlier, *T. chironomi* is considered to be distinct from these three species.

Full treatment of the taxonomic status of the other ciliates involved is beyond the scope of the present paper. A summary of my present conclusions concerning these, as well as the more firmly established forms, is available in Table 1. It will be noted that *T. chironomi* is occasionally involved as a possible synonym. Should future work permit any such questionable possibility to become a reality, then the most recent name(s), including *T. chironomi*, would have to fall as synonym(s) of the oldest proposed name. It is my belief, at the present time, that there is little danger of the occurrence of this.

Table 1. *Ciliates probably assignable to the genus* Tetrahymena *found in nature as facultative or accidental parasites*

Specific name originally used	Reference	Host; location of infection; source of material	Comments	Suggested systematic status
None	Lamborn (1921)	Mosquito, *Aëdes* (*Stegomyia*) *Scutellaris* (actually *A.* (*S.*) *albopictus*?); in body cavity and anal gills of larvae; from earthenware pot, Kuala Lumpur, Federation of Malaya	Studied cytologically by Keilin (1921). Facultative parasitism; infection fatal	*Tetrahymena stegomyiae*
Lambornella stegomyiae	Keilin (1921)	As above. Cysts on cuticle of host, but mode of entry postulated as via mouth	Considered *Glaucoma pyriformis* by Wenyon, Lwoff, Muspratt, and others. As above	As above
None	MacArthur (1922)	Mosquito, *Culiseta* (*Theobaldia*) *annulata*; in body cavity, especially head region, of larvae; from field dyke near Blackpool, England	More morphological data needed. Studied cytologically by Wenyon (1926). Not '*Lambornella stegomyiae*', according to Keilin in MacArthur (1922). Facultative parasitism; infection fatal	*T. pyriformis*? *T. chironomi*?
Glaucoma parasiticum	Penard (1922)	Amphipod, *Gammarus pulex*; on and in gills; from Thonex, near Geneva, Switzerland	In need of rediscovery and redescription. Penard thought it close to '*Glaucoma pyriforme*'. Facultative or accidental parasitism? 'Infection' not fatal	*T. parasitica*
G. piriformis	Treillard & Lwoff (1924)	Midge, *Chironomus plumosus*; in body cavity of larvae; from environs of Paris	Exhibited conjugation in host. Facultative parasitism; infection fatal	*T. chironomi*
Balantidium knowlesii	Ghosh (1925)	Midge, *Culicoides peregrinus*; in 'coelomic cavity' (of larva?); from Calcutta, India	Description *very* inadequate. Accidental parasitism only? Infection fatal?	*T. pyriformis*? *T. chironomi*? *T.* sp.??
Glaucoma sp.	Epstein (1926)	Bream, *Abramis brama*; in gut,	More morphological data needed.	*T. corlissi*?

		from Calcutta, India	Infection fatal?	*T.* sp.??
Glaucoma sp.	Epstein (1926)	Bream, *Abramis brama*; in gut, vascular system, and spinal canal of 7 mm. embryos; from aquarium in Moscow	More morphological data needed. Amicronucleate? Facultative parasitism? Infection fatal	*T. corlissi*? *T. faurei*? *T. pyriformis*?
G. pyriformis	Wenyon (1926)	See MacArthur (1922), above	Inadequate description. No cuticular cysts observed. Facultative parasitism; infection fatal	*T. pyriformis*? *T. chironomi*?
Protobalantidium knowlesii	Abe (1927)	See Ghosh (1925), above	A new genus is proposed for Ghosh's poorly described '*Balantidium*'	*T. pyriformis*? *T. chironomi*? *T.* sp.??
Leptoglena knowlesii	Grassé & de Boissezon (1929)	See Ghosh (1925), above	Another new genus is proposed for Ghosh's unrecognizable form!	*T. pyriformis*? *T. chironomi*? *T.* sp.??
Turchiniella culicis	Grassé & de Boissezon (1929)	Mosquito, *Culex* (*Culex*) *pipiens*(?); in haemocoele of one adult ♀; from Villenouvelle (Haute-Garonne), France. Parasites 'normally' in larvae?	Said to possess '22–24 lines of cilia'; post-oral meridians missed? Only sectional material (of host) studied. Exconjugants seen? Accidental parasitism only? Infection not fatal	*T. chironomi*? *T. pyriformis*??
T. culicis	de Boissezon (1930)	As above. Ciliates escape from dying adult in water to infect larvae?	As above. No new data	As above
Paraglaucoma rostrata	Kahl (1931)	Rotifer, unidentified; in tissues of decomposing body; from moss in Germany	Cysts found in rotifer lorica. Histophagous, edaphic species (Kahl, 1926). More likely scavenger than parasite in this case?	*T. rostrata*
P. limacis	Warren (1932)	European slug, *Deroceras reticulatum*; in liver and faeces; from Natal, South Africa. Also possibly in nearby soil	Some reconcilable errors in description: cf. Kozloff (1946, 1956*b*, *c*). Facultative parasitism; infection not fatal	*T. limacis*

Table 1 (*cont.*)

Specific name originally used	Reference	Host; location of infection; source of material	Comments	Suggested systematic status
Glaucoma paedophthora	van den Berghe (1934)	Fresh-water snails, *Planorbis* sp. and *Physopsis* sp., in egg masses, killing embryos; from Elizabethville, Belgian Congo	More morphological data needed. Amicronucleate? Accidental parasitism only? 'Infection' fatal	*T. pyriformis*? *T. chironomi*? *T. parasitica*?
Glaucoma sp.	Shumway (1940)	Salamander, *Ambystoma texanum*; in central nervous system of 12 mm. lab.-raised larva; from vicinity of Urbana, Illinois	More morphological data needed. 'Approximately 20 longitudinal rows of cilia'. Studied only in sectioned material of host. Accidental parasitism? Infection fatal?	*T. pyriformis*? *T. corlissi*??
Tetrahymena geleii	Knight & McDougle (1944)	Plymouth Rock cockerels; in infra-orbital and other sinuses, digestive tract, etc., of eight 9-week-old chicks; from Columbia, Missouri	Identified after additional study of cultured forms, assumed identical, taken from nearby stagnant pool. Accidental, freakish case of parasitism? Infection not fatal	*T. pyriformis*
None	Muspratt (1945)	Tree-hole breeding culcine mosquitoes: *Aëdes* (*Aëdimorphus*) *haworthi*, *A.* (*A.*) *marshalli*, *A.* (*Finlaya*) *fulgens*, *A.* (*Stegomyia*) *aegypti*, *A.* (*S.*) *calceatus*, *A.* (*S.*) *metallicus*, *Culex* (*Culex*) *decens*, *C.* (*Culiciomyia*) *nebulosus*; generally restricted to body cavity and anal papillae of larval stages, occasionally found in adult; from Livingstone, Northern Rhodesia		As below
Glaucoma pyriformis	Muspratt (1947)	As above. *A. fulgens* considered most suitable host in nature. Cuticular cysts involved in entry through larval body wall	Considered by Muspratt as probably identical with Keilin's (1921) '*Lambornella stegomyiae*', although little morphological data given. Facultative parasitism; infection fatal	*T. stegomyiae*
Tetrahymena limacis	Kozloff (1946)	European slug, *Deroceras reticu-*	Well described, but at one time	*T. limacis*

Tetrahymena limacis	Kozloff (1946)	European slug, *Deroceras reticulatum*; in liver and other visceral organs; from Berkeley, California, and Tillamook and Portland, Oregon	Well described, but at one time considered not identical with Warren's '*Paraglaucoma limacis*' by Mugard, Corliss, and others. Facultative parasitism; infection not fatal	*T. limacis*
T. limacis	Borden (1948—unpublished)	European slug, *D. reticulatum*; in liver (and faeces?); from Charlottesville, Virginia	*Colpoda steinii* more plentiful in her slugs. Report of 'cysts' suggests to Thompson (1958*b*) that *T. rostrata* also probably present in some cases. Facultative parasitism: infection not fatal	*T. limacis* (and *T. rostrata*?)
Tetrahymena sp., *T. faurei*	Corliss (1951), 1952*a*, *b*, *c*, 1953*a*)	Trout, *Salmo irideus*; in tail musculature and spinal canal of single 15 mm. embryo; from laboratory aquarium in Paris. Entry through caudal injury	More morphological and physiological data desirable; taxonomic status has become uncertain. Accidental parasitism only? Infection fatal?	*T. faurei*
Balantidium sp., *Tetrahymena* sp., *T. corlissi*	Speidel (1951–58)	Amphibians: *Bufo*, *Rana*, *Pseudacris*, *Pseudotriton* spp.; in fluids and tissues of poorly nourished larvae; from Woods Hole, Massachusetts and Charlottesville, Virginia	Early descriptions inadequate; assigned to genus *Tetrahymena* by Thompson & Speidel (1955); subsequently recognized as *T. corlissi*. Facultative parasitism; infection fatal?	*T. corlissi*
Balantidium-like ciliate	Seamster (1952)	Man; in spinal canal of 6-year-old boy; from Corpus Christi, Texas. Postulated to have come from colon via lymphatic channels	Inadequate description. Size, shape, and activity of organism suspiciously *Tetrahymena*-like. No early examination made of faeces; later, negative. Accidental, freakish case of parasitism? Infection not fatal (cleared up by terramycin, administered intravenously)	*T. pyriformis*? *Balantidium coli*?

Table 1 (*cont.*)

Specific name originally used	Reference	Host; location of infection; source of material	Comments	Suggested systematic status
Tetrahymena parasitica	Corliss (1952*b*, *c*, 1953*a*)	Midge, *Chironomus plumosus*; extremely numerous in body cavity of larvae; from environs of Paris. (Also see below)	Provisionally assigned to same species as Penard's '*Glaucoma parasiticum*', in spite of inadequacy of his description. Facultative parasitism; infection fatal	*T. chironomi*
T. pyriformis	As above	As above. Always in mixed infections, with above species in predominance	No doubt as to identity. Amicronucleate. Never found alone in the chironomid larvae. Facultative or accidental parasitism?	*T. pyriformis*
Glaucoma parasiticum?	Canella (1954)	Rotifer, *Brachionus calyciflorus*; in moribund specimen; from fresh-water source, Ferrara, Italy	Limited description. Histophagous scavenger, unlikely exhibiting parasitism of any kind	*T. rostrata*?
Tetrahymena rostrata	Stout (1954)	Enchytraeid oligochaete; throughout body tissues; from soil and litter, Lower Hutt, New Zealand. Entrance through setal follicles	Well described. Also found free-living in soil and litter. Edaphic, histophagous species. Facultative parasitism; infection fatal	*T. rostrata*
Tetrahymena sp.	Corliss (1954*a*, and unpublished)	Mosquito, *Wyeomyia* (*Wyeomyia*) *smithii*; in body cavity of larvae; from pitcher plants, Bethany Bog, Connecticut	No doubt as to identity. Amicronucleate. Facultative or accidental parasitism? Infection fatal	*T. pyriformis*
Tetrahymena sp.	Corliss (1954*a*, and unpublished)	Salamander, *Ambystoma maculatum*; in egg masses and developing embryos; from Hanover, New Hampshire	Identity quite certain, although limited observations. Amicronucleate. Accidental parasitism? Infection fatal?	*T. pyriformis*
Tetrahymena sp.	Corliss (1954*a*, and unpublished)	European slug, *Deroceras reticulatum*; in testicular tissues; from	Identity quite certain, although studied only in sectioned host	*T. rostrata*

		Hampshire	tism? Infection fatal?	
Tetrahymena sp.	Corliss (1954*a*, and unpublished)	European slug, *Deroceras reticulatum*; in testicular tissues; from vicinity of Dalhousie, Nova Scotia	Identity quite certain, although studied only in sectioned host material. Facultative parasitism; infection probably not fatal	*T. rostrata*
Lambornella sp.	Nance (1954)	Guppy, *Lebistes reticulatus*; throughout body tissues, especially musculature; from commercial source: Beldt's Aquarium, Robertson, Missouri	Description not published. Unpublished studies by Dr Ruth McClung Jones and colleagues show it to be a strain of *T. corlissi* (personal communication). Facultative parasitism; infection fatal	*T. corlissi*
Tetrahymena sp.	Burns *et al.* (1955)	As above	As above	As above
T. corlissi	Thompson (1955*a*)	Salamander, *Pseudotriton* sp.; in circulatory system of a larva; from Charlottesville, Virginia	Adequately described as a new species. Facultative parasitism; infection fatal?	*T. corlissi*
Tetrahymena sp., *T. pyriformis*?	Nigrelli, Jakowska & Padnos (1955, 1956)	African lungfish (*Protopterus annectens*), kissing gourami (*Helostoma temmincki*), platyfish (*Platypoecilus variatus*), guppy (*Lebistes reticulatus*); on or in skin, gills, and surface wounds, occasionally in body cavity; from aquaria in New York City	No morphological description offered. 'Pathogenic epibiont ...for fish in a closed water circulation'. Facultative parasitism? Infection fatal?	*T. corlissi* and/or *T. pyriformis*
Paraglaucoma rostrata	Gellért (1956, 1957)	Rotifer and tardigrade, unidentified; in deteriorating bodies; in moss-turf on rocks from Tihany, Hungary	Limited description. Edaphic species, histophagous scavenger. Unlikely a parasite in these cases	*T. rostrata*
Tetrahymena limacis	Kozloff (1956*b*, *c*)	European slug (*Deroceras reticulatum*), native slug (*Prophysaon andersoni*), native snail (*Monadenia fidelis*); in liver and alimentary tract; from localities near Oswego and Portland, Oregon	Well described. Interesting transfaunation experiments carried out. Facultative parasitism; infection not fatal	*T. limacis*

Table 1 (*cont.*)

Specific name originally used	Reference	Host; location of infection; source of material	Comments	Suggested systematic status
T. rostrata	Kozloff (1957)	European slug, *Deroceras reticulatum*; in renal organ; from one locality in Oswego, Oregon	Well described. Encystment noted. Facultative parasitism; infection not fatal	*T. rostrata*
T. limacis	Dobrzańska (1958)	Fresh-water clams, *Pisidium casertanum*, *P. obtusale*, *Sphaerium lacustre*; in mantle cavities and on gills; from several small bodies of water near Mikolajki, Poland	No morphological description published. Ciliates in the lamellibranchs only during unfavourable periods in the ponds. Temporary association, perhaps best not considered parasitism at all. 'Infection' not fatal	*T. limacis*?
T. pyriformis	Dobrzańska (1958)	Clams, as above, but in *Sphaerium corneum* rather than *S. lacustre*	As above	*T. pyriformis*?
T. limacis	Kazubski (1958)	Land snail, *Trichia lubomirskii*; in liver; from vicinity of Duszatyn, near Sanok, Poland	Well described. Facultative parasitism; infection not fatal	*T. limacis*
T. rostrata	Kazubski (1958)	Land snail, *Zonitoides nitidus*; in renal organ; from ponds in Julin, near Warsaw, Poland	Well described. Facultative parasitism; infection not fatal	*T. rostrata*
T. corlissi?	Speidel (1958*a*)	Catfish, *Ameiurus* sp.; especially in developing central nervous system of very young embryos; from fresh-water pond in Falmouth, Massachusetts	More morphological data needed. Amicronucleate. Very similar to *T. corlissi*, according to Speidel. Facultative parasitism? Infection fatal?	*T. corlissi*? *T. faurei*? *T. pyriformis*?
T. rostrata	Thompson (1958*a*, *b*)	European slug, *Deroceras reticulatum*; in faeces of single specimen, from Culpeper, Virginia	No doubt as to its identity. Cysts repeatedly passed in faeces. Facultative parasitism; infection not fatal	*T. rostrata*

		Virginia	infection not fatal	
T. pyriformis	Laird (1959)	Mosquitoes: *Aedes* (*Stegomyia*) *albopictus*, *Culex* (*Culex*) *fuscocephalus*, *C.* (*C.*) *gelidus*, *C.* (*C.*) *taeniorhynchus trisiamensis* = *C.* (*C.*) *t. summorosus*; in larvae; from Singapore	Studied mainly from haematoxylin preparations. Used MacArthur's (1922) work for comparison	*T. pyriformis*?
T. pyriformis	Lom (1959)	Oligochaete, *Allolobophora caliginosa* var. *trapezoides*; coelomic cavity; from soil, near Prague, Czechoslovakia	Briefly described. Number of ciliary meridians and size of micronucleus make its exact systematic status unclear	*T. pyriformis*? *T. chironomi*? *T. parasitica*? *T.* sp.??
T. limacis	Windsor (1959)	European slug, *Deroceras reticulatum*; in liver and possibly other organs; from vicinity of Urbana, Illinois	No doubt as to its identity. *Colpoda steinii* also found, but not from same host specimens. Facultative parasitism; infection not fatal	*T. limacis*
T. stegomyiae comb.nov.	Corliss (present paper)	Mosquito, *Aëdes* (*Stegomyia*) *metallicus* and *A.* (*S.*) *aegypti*; in body cavity and on cuticle of larvae; from mid-eastern Transvaal, South Africa. (Hosts with ciliates fixed by Dr J. Muspratt)	See text (§V). Adequate data to conclude that Keilin's and Muspratt's ciliates were identical. Facultative parasitism; infection fatal	*T. stegomyiae*
T. chironomi sp.nov.	Corliss (present paper)	Midge, *Chironomus plumosus*; extremely numerous in body cavity of larvae; from environs of Paris (and from carp ponds near Berlin)	See text (§§III–VI). Lethal selfing type of conjugation one of most interesting features. Facultative parasitism; infection fatal	*T. chironomi*

VI. CONSIDERATION OF HOST–PARASITE RELATIONSHIPS

A. *General considerations*

Definition of terminology. In a broad sense facultative parasitism refers to the ability of an organism which normally leads a wholly free-living existence to thrive for a time in or on another organism. If one considers parasitism to be a subcategory of the more general phenomenon of symbiosis, separated from mutualism and commensalism primarily on the basis of the effect on the host, then facultative parasitism (also) should imply some deleterious effect on the host involved. For the protozoa this immediately excludes the majority of episymbiotic forms which thus will not be considered in the present section. In a narrower sense, such as that adopted by Kirby (1941, p. 895), facultative parasitism may be considered to require that the parasitism 'is a natural occurrence in the bionomics of the species'. Then the terminology 'accidental parasitism' should be applied to 'unnatural' instances, such as cases in which a free-living organism just happens to obtain entrance into a host, quite by accident, but thrives there nicely because of its adaptability to such a medium. One would not expect to find regular, repeated occurrences of such associations of a given host with the same species of free-living protozoon.

As Kirby agrees, often it is difficult to separate accidental and facultative parasitism in practice. There is possibly a third type of relationship which, again, cannot always be distinguished categorically from the other two. Histophagous protozoa and scavengers often attack moribund or wounded, as well as dead organisms, entering bodies through accidental mutilations, openings caused by deterioration of certain structures, etc. Occasionally these cases may be reported as facultative parasitism, and, indeed, some of the same species may be involved. In the present paper such instances known to me are included in the very general, over-all category of facultative parasitism *sensu lato*.

Natural occurrence of ciliates as facultative or accidental parasites. The prerequisites for any free-living ciliate to live successfully for a longer or shorter period of time within the body of another organism are simple: it must be available in abundance in the same ecological niche as the potential host; it must be preadapted to life in the medium offered by the fluids and tissues of the host's body; it must find a suitable mode of entry into the body under natural conditions. Actually these requirements are very demanding and apparently are not easily met by the majority of free-living fresh- or salt-water species of ciliated protozoa.

Various members of the hymenostome genus *Tetrahymena* appear to meet the conditions set forth above. It should be pointed out, however, that ciliates belonging to genera in quite separate families also have been found associated endosymbiotically with insect larvae and occasionally other invertebrate animals, such as slugs and crabs. But such cases are rare in the literature and need not concern us further here. Direct reference to them may be found in the reviews by Kirby (1941) and Mugard (1949).

Types of hosts. Vertebrates as well as invertebrates have been implicated as hosts for species of facultatively (in the broadest sense) parasitic tetrahymenae.

Aquatic forms, often larval stages, obviously are most susceptible to such infection, but there are exceptions. Specific consideration of hosts is treated in the appropriate following sections; also Table 1 (pp. 130–7) may be consulted for an indication of the variety of forms which have been involved to date.

B. Tetrahymena chironomi *and* Chironomus plumosus

Chironomids as hosts in general. Chironomid larvae, it should be mentioned, long have been known to harbour parasites or commensal symbionts of various kinds (see especially Thienemann, 1954). In the body cavity, in parts of the alimentary tract, or in tissues and cells of their several major organs have been found many species of nematodes, trematodes, leptomonad flagellates, gregarine telosporidians, and microsporidians. On the outside, as episymbionts or as true parasites, stalked peritrich ciliates and water-mite larvae are found in abundance. Incidentally, a protozoon commensal in the digestive system which generally is overlooked by reviewers is the small *Amoeba chironomi* described by Porter (1909) 50 years go. Although I made no attempt at careful study of the organism, I believe that I encountered this amoeba once myself in a single specimen of *C. plumosus* dissected in the winter of 1952.

Although ciliated protozoa occasionally have been reported from body cavities of mosquitoes (see below), the only published case involving midges, other than Treillard & Lwoff's (1924) and my own, is the short note by Ghosh (1925), working in India, of '*Balantidium knowlesii*' from a larval stage of *Culicoides peregrinus*. But this host is not a member of the family Chironomidae, and it is impossible to be sure of the exact identity of the ciliate involved.

Unpublished findings of hymenostome ciliates in true chironomids include observations, transmitted to me as personal communications, by Barbara Walshe (now Maetz) in about 1946 from larvae collected in the London area; by E. Fauré-Fremiet in 1949 from material taken at Juine, near Paris; and by D. Barthelmes in 1957 and 1958 from many larvae found in ponds near Berlin. A. A. Johnson, in about 1953, noted 'small ciliates in abundance' in several specimens of *Chironomus* sp. from Douglas Lake, Michigan, but no attempt at identification was made (personal communication). There is no doubt but that the *T. chironomi–C. plumosus* association represents a bona fide case of true facultative parasitism.*

The following paragraphs refer only to my own Parisian material studied in 1951–52, unless otherwise stated.

Incidence of infection. From mid-January to mid-April, 1952, a total of 2149 specimens of *C. plumosus*, principally in the fourth instar larval stage, were examined. Of these, 186 contained ciliates, giving an over-all infection figure of 8·7%. This figure, however, is misleading. It is probably too low, since in the early days of my discovery of ciliates in these chironomids I was selecting only the

* While this paper was in press, Dr A. A. Johnson generously sent me some fixed specimens of infected chironomid larvae (*C. plumosus*) which he had obtained from Douglas Lake, Michigan, in the summer of 1957. I am indebted to Mr Donald A. Windsor for making cytological preparations of the material adequate for identification of the ciliates as members of the species *T. chironomi*.

largest, most healthy-appearing larvae because of the greater desirability of their intestines as food for other protozoa being cultured at the time. Originally, smaller, paler forms were not even examined. There was considerable variation in the incidence of infection in different packets of the chironomids, also. This observation is supported by data from selected examples presented in the accompanying tabulations (Tables 2 and 3). It is also interesting to note that Treillard & Lwoff (1924) reported thirteen parasitized chironomids in one lot of 300 larvae, but none in a second lot of the same size.

Table 2. *Variation in percentage of ciliate infection in representative lots of chironomid larvae*

Total no. of larvae examined from given lots	No. containing ciliates	Incidence of infection (%)	Comments
300	4	1·3	Large (16–24 mm.) larvae.
77	27	35·1	Small (< 12 mm.) larvae from same lot
115	10	8·7	
70	1	1·4	
150	3	2·0	
135	16	11·8	Generally large larvae
63	2	3·2	
34	0	0·0	
36	2	5·6	

Table 3.* *Relationship of percentage of ciliate infection to larval stage of chironomid host*

Total no. of larvae examined	No. containing ciliates	Incidence of infection	Larval size and instar stage
34	0	0·0	23 mm., 4th (advanced)
92	5	5·4	19–21 mm., 4th (early)
106	24	22·6	15–18 mm., 3rd (advanced)
88	38	43·2	13–15 mm., 3rd (early)
4	2	50·0	7–8 mm., 2nd

* Prepared from data kindly supplied by Dr Maetz who worked with some of my material in Paris in 1952.

A curious fact, briefly alluded to earlier in this paper, which affects the true incidence of infection of *C. plumosus* by *T. chironomi* is the presence of an amicronucleate *T. pyriformis* within some of the same host larvae. In other words, the infection was often a double one in my Parisian material: the body cavity of a single specimen of the host organism would contain, simultaneously, teeming populations of two species of tetrahymenid ciliates. Under the ordinary powers of magnification possible with a dissecting microscope it was practically impossible to distinguish between these organisms; unfortunately, in only one-fourth of the 186 cases of ciliate-infected chironomids was exact identification, employing proper cytological techniques (see Figs. 6–7), ever made. But from this sample of cases

the following interesting tentative conclusions can be drawn: *T. pyriformis* was never found as the sole parasite in the chironomids examined; *T. chironomi*, on the other hand, was discovered alone in about half of the cases; in double infections carefully observed, *T. chironomi*, the smaller species, greatly outnumbered the larger species, *T. pyriformis*.

With regard to numbers of ciliates per host, no exact count ever was made. A reasonably conservative estimate for a heavily infected fourth-instar larva, however, is 100,000–200,000 ciliates. A very light infection in a large chironomid could give rise to an extremely dense population of ciliates in 48 hr. Although the posterior end of the host's body ordinarily seems to be preferred, in heavy infections the entire haemocoele, including the pre-anal papillae and all gills, is crammed full of wriggling protozoa: an impressive sight to behold (see Figs. 1–3). The great number of parasites per host is stressed, since it is much higher than the figures given in other reports of facultative parasitism of tetrahymenid ciliates in a variety of hosts.

Effect on the host. There is no doubt but that the presence of ciliates, in sufficient abundance, eventually causes the death of the chironomid. Treillard & Lwoff (1924) state that this occurs within 8 days after postulated entrance of the protozoon. In the present work it was found that the duration of the infection could be much longer; e.g. one lightly parasitized 15 mm. larva remained alive for 41 days after it was discovered to be infected. If a population of ciliates in the haemocoele becomes large quickly, death seems to occur in a short time; but if the larva is able to utilize an apparently inherent capacity to inhibit multiplication of the parasites, the infection may remain more or less static for some time. Large-scale experiments were not carried out on these problems; yet it is interesting to note that most of the non-parasitized chironomids selected for controls and kept under the same laboratory conditions as the parasitized, destined-to-die larvae, reached pupal and adult stages successfully.

Exactly how the facultative parasites cause the death of their insect hosts is unknown. The bodies of active ciliates in the body cavity are filled with pigmented yellowish brown granules which Treillard & Lwoff (1924) suggest are derived from chironomid haemoglobin. Also it is likely that host fat bodies are utilized by the tetrahymenae, as has been shown in experimental work with other insect larvae inoculated with a strain of *T. pyriformis* (Lwoff, 1924; Janda & Jírovec, 1937). I never have seen any evidence of the protozoa attacking the digestive tract of the host.

Dr Maetz kindly ran exploratory tests on the metabolism of nine large infected larvae (19 mm length) in glass U-tubes, following her standard techniques (for example, see Walshe, 1947, 1950). An average of 222 mm.^{3} of oxygen per gramme (wet weight) per hour was obtained, whereas the average of control (non-parasitized) animals of similar size is about 200 mm.^{3}. However, this apparent increase of metabolism is not statistically significant, since it falls within the range known for normal larvae. Further experimentation, which might show it to be significant, remains to be done.

Larvae of any size apparently can become infected; how this may occur is

discussed below under 'Possible modes of entry'. As a general rule, the smaller the larva the longer it can remain alive with the infection. Small ones, in the 13–15 mm. size range, can survive from 2 to 41 days after initial detection of the presence of the parasites, often with the infection remaining light. Large ones, around 20 mm., often are seen to succumb, with a very high number of ciliates within them, in 1–6 days from starting time of the observations. Infected larvae never succeed in pupating, nor has a laboratory-raised chironomid pupa ever been found harbouring tetrahymenae.

Effect on the parasite. The larval chironomid body cavity seems to afford a perfect culture chamber for tetrahymenid ciliates. The discovery of the association between *T. chironomi* and *C. plumosus* in widely separated geographic locations as well as in the same general location (Paris) in periods nearly 30 years apart leads one to suspect that infection of such insect larvae is a natural occurrence in the bionomics of strains of the ciliate species under present consideration.

One startling and curiously discordant fact remains as a perplexing problem, however. Having consumed various nutritive substances from their host, multiplying rapidly all the while, and having brought their host's life cycle nearly to an end, the ciliates then all enter into an act of sexuality which spells their own doom! Practically 100 % of the ciliates (not including the amiconucleate *T. pyriformis* which may have been present in the same larval chironomid) are thus destined to die, by virtue of the highly fatal kind of selfing conjugation which they undergo (see §IV, above), as soon as or shortly after the death of the similarly ill-fated host.

It should be stressed here that the 'life cycle' described for the ciliates *within the insect's body* does not imply that this is the *total* life cycle or that a parasitic existence is an obligate one for *T. chironomi*. It is true that exconjugants, following selfing, perish in depression slide cultures; but also they do so if left within the body of the dying host. On the other hand, ciliates released in a preconjugating stage from the chironomid thrive in laboratory culture, continuing to feed (on bacteria), divide, etc., until onset of the always fatal act of conjugation. Furthermore, not only is it very likely that the *T. chironomi* entering chironomid larvae in nature come from a free-living life in the same habitat as the insect, but several conspecific strains of this species have been found in bodies of water not particularly known to have contained any chironomids at all at the time of collection of the water sample containing the ciliates.

Possible modes of entry. This topic receives further consideration below; here we are concerned solely with the case of *T. chironomi* (and occasionally *T. pyriformis*) as parasite and *C. plumosus* as host.

There are probably only four possibilities for entry of this ciliate into the haemocoele of chironomid larvae, and three of these seem unlikely. Entrance via the digestive tract would appear to be ruled out for several reasons: ciliates are never found in the alimentary canal; the intestinal wall seems intact; experiments designed to demonstrate the possibility of this mode of entry gave negative results. Rich cultures of protozoa (primarily strains of *T. pyriformis*) placed in containers with parasite-free larvae of various sizes and ages had no effect on the chironomids

which lived as long and well as controls and never became infected. The protozoa did not succeed in entering the haemocoele via the digestive tract, although hundreds of them must have been ingested. Janda & Jírovec (1937), some years ago, also demonstrated that infection by way of the mouth was impossible in their extensive experiments with an amicronucleate strain of *T. pyriformis* and a number of insect larvae, including *Tipula* sp.; direct inoculation of the ciliates into the haemocoele, however, was highly successful, as other workers have shown, too (Lwoff, 1924; Thompson, 1958*a*; McLaughlin, 1959).

Direct penetration through the intact body wall also is unlikely. This did not occur in the experiments cited above; and it may be noted that the cuticular layers of chironomid larvae are quite thick: 7μ or more, according to Wigglesworth (1950). Furthermore, the species of *Tetrahymena* involved are not particularly voracious tissue-feeders, as are some other species in the genus, and would not be apt to attack the larvae directly. Entrance through accidental, artificial breaks is plausible except for the fact that the incidence of such wounds among chironomids in nature would appear to have to be rather high to explain the observed incidence of infection. None of the parasitized larvae examined seemed to show evidence of having or of having had external wounds.

Until further experimental work is carried out, the exact mode of entry must remain unknown. The fourth possible method, and the one which seems to me to be the most likely in the present case, however, is invasion of the larvae through more or less 'natural' breaks or weaknesses which would be present during the moulting process. The temporary quiescence of the chironomid immediately following the shedding of its old cuticle would tend to favour entrance of the free-living ciliates present in the surrounding water, too. Perhaps the region of the hooks on the pre-anal papillae is most susceptible to penetration, especially if minute temporary openings or breaks should occur there with some frequency at the time of or just following the process of moulting. It is pertinent to mention that workers studying mermithid infections of *C. plumosus* and related species have found this posterior area of the larval body to be the region of penetration by the nematodes (for example, see Comas, 1927; Svabenik, 1928).

That only a single ciliate enters each host was postulated by Treillard & Lwoff (1924). There is no doubt but that one organism could establish itself in the rich medium offered by the fluids of the larval chironomid and give rise to a heavy population in a short time. Furthermore, one would suspect that the odds are against multiple infection in nature, since this probably would require the presence of many ciliates at exactly the right place at exactly the right time. Finally, since the conjugation observed is of a selfing kind (see §IV, above), simultaneous entry of ciliates belonging to both members of a pair of complementary mating types is not required to explain the occurrence of the sexual phenomenon. On the other hand, the frequent finding of both *T. chironomi* and *T. pyriformis* in the same host specimens is adequate proof that at least *two* ciliates originally entered those particular larval chironomids. Thus Treillard & Lwoff's general hypothesis may be considered to be invalid.

C. *Other species of* Tetrahymena *and their hosts*

The case of the type species, Tetrahymena pyriformis. As inspection of Table 1 (pp.130–7) will reveal, this species is justifiably implicated fairly often among the reported incidences of tetrahymenae in both vertebrate and invertebrate hosts. In view of the ubiquitous occurrence of *T. pyriformis* and its ready adaptability to different cultural environments, including non-particulate media, it is not surprising that this small ciliate should be discovered in more or less close association with a variety of other organisms in nature (Corliss, 1954*a*). On the other hand, it is obvious that the conclusion of some earlier reviewers, that all cases of ciliated protozoa found as facultative parasites must be assigned to this species alone, is entirely indefensible.

It has been demonstrated by experimentalists that *T. pyriformis* can thrive well following inoculation into the body cavity of a number of hosts, particularly insect larvae (Janda & Jírovec, 1937; Lwoff, 1924; McLaughlin, 1959; Thompson, 1958*a*). The hosts generally die of the infection. Yet the method of penetration in nature has remained a mystery, even as the mode of entry of this species into slugs, in experiments carried out by Kozloff (1956*a*), still rests uncertain (although probably through the mouth in this particular case). In fact, I am inclined to believe that most of the cases of 'parasitism' involving *T. pyriformis* actually are accidental in occurrence, with entrance permitted through wounds or freakish breaks or other kinds of artificial openings. That these ciliates are available in the 'host's' environment, are attracted to the specific situations, and can do well upon having gained entrance probably explains the more frequent discovery of this species than other holotrich ciliates, under such conditions. Thus clams with open mantle cavities, wounded mosquito or other insect larvae or adults, injured amphibians or fishes, unusually susceptible or somehow freakishly exposed young birds (or even a young man) can be envisioned falling readily as 'prey', rather than 'host', to a population of *T. pyriformis* in their vicinity (see appropriate references in Table 1). The case of the amicronucleate members of this species in *C. plumosus*, however, is probably of a different sort.

Unlike certain other species of the genus, *T. pyriformis* generally is not considered to be a cyst-former (Corliss, 1953*a*, 1954*a*; Weis, 1954). Watson (1946), however, observed them in what we now know to have been an amicronucleate strain of *T. pyriformis*, and, very recently, Hurst (1957) has reported them in certain strains of variety 2 of this species. It remains unlikely that cysts are involved in any of the cases of 'facultative' parasitism reported to date. In this regard, Watson's experimental attempt to infect mice with *Tetrahymena* by feeding them great numbers of cysts on bread is interesting: no infections resulted, nor were any cysts recovered from the faeces. Incidentally, McCaul (1956) who had no cysts to work with, also failed to infect small mammals with this species, using a variety of ways of introducing the ciliates into parts of the body.

The case of Tetrahymena stegomyiae *and mosquitoes.* Here we seem to have a genuine example of facultative parasitism; in fact, some protozoologists consider that obligate parasitism is indicated. Yet the fact that the ciliates, if released from

a larval host, live well in laboratory culture quite free from the host appears to weaken that argument. Isolation of these organisms as free-living forms from waters not containing mosquito larvae at the time would serve as a desirable final proof. The once-controversial matter of the cuticular cysts, so readily formed on the outside of larval bodies exposed to populations of the ciliate both in nature and in the laboratory, now may be considered as settled (see §V, above). They are an integral part of the life cycle of *T. stegomyiae* and very likely do afford the means of penetration through the body wall into the haemocoele of the host. This is true of no other species of *Tetrahymena*.

T. stegomyiae kills its host. In this respect it resembles the relationship of *T. chironomi* to chironomid larvae, discussed in detail in the preceding section of this paper. Purely accidental instances of invasions of 'hosts', as mentioned for *T. pyriformis*, above, may or may not give rise to fatal infections. On the other hand, some truly facultative parasites apparently bring no great harm to their hosts: *T. limacis* and *T. rostrata* represent this group (see below).

There are a number of problems concerning this ciliate from culicine mosquitoes which have not been answered in the limited investigations of Lamborn (1921), Keilin (1921), Muspratt (1945, 1947) and myself (see §V). Additional cytological data are needed as well as information of a more experimental nature, such as the reactions of the protozoa to transfaunation into other hosts. All such work, which may be of considerable significance in systematics of the genus and in understanding host-parasite relationships in facultative parasitism in general, must await re-availability of the material. In the meantime it must be remembered that all ciliates found in mosquitoes are not *a priori* members of the species *T. stegomyiae*: consult Table 1 and see footnote p. 126.

The case of the 'limacis-rostrata' *group.* Two of the thirteen species which I consider as belonging to the genus *Tetrahymena* are somewhat set apart from all the others by their choice of ecological niche. *T. rostrata* especially, and *T. limacis* apparently (more work needs to be done here), are edaphic species, found free-living in soil, soil and forest litter, and moss-lichen habitats. (*T. pyriformis* and others only occasionally may be isolated from such environments.) In the facultatively parasitic phase of the life histories of these two species the hosts involved are the very organisms one might have suspected: enchytraeid worms, snails, slugs, and possibly rotifers and tardigrades (see appropriate references in Table 1).

T. limacis and *T. rostrata* do not kill their terrestrial pulmonate gastropod hosts (see especially the extensive investigations by Kozloff, 1946, 1956*b*, *c*, 1957), although Stout (1954) reported the enchytraeid infections with *T. rostrata* to be fatal. Transfaunation experiments have been carried out successfully by Kozloff. In the case of slugs and snails, the portal of entry for the edaphic protozoa in nature is quite likely by ingestion with food, particularly food contaminated with faecal material from other infected individuals. For the enchytraeid oligochaetes studied by Stout the place of entry appears to be through setal follicles at times when setae are discarded or lost.

To date *T. rostrata* has been found more often independent of hosts, e.g. in moss

samples, than associated directly with other organisms (Kahl, 1926, 1931; Corliss, 1952*c*, *d*; Stout, 1954; McArdle, 1959). Its discovery inside the carcass of certain small edaphic invertebrates probably reflects its histophagous tendencies as a scavenger more than its ability to exhibit facultative parasitism (Kahl, 1931; Canella, 1954; Gellért, 1956, 1957). On the other hand, it has been found in slugs, snails, and enchytraeid worms as well, as mentioned previously (Stout, 1954; Kozloff, 1957; Kazubski, 1958; Thompson, 1958*a*, *b*). This should serve as a caution to future investigators of the gastropod hosts in particular: a single individual might well be found to contain simultaneously *T. rostrata* and *T. limacis*, and possibly *Colpoda steinii*, a trichostome ciliate common in slugs from some areas (Reynolds, 1936; Borden, 1948; Windsor, 1959), as well!

T. limacis, I would agree with Kozloff (1956*c*), seems to be primarily a parasite. It is very possible that the ciliates spend short periods outside the host, probably voided with the faeces, allowing infections to spread in a population of the gastropods in a given area. Kozloff (1946) was unable to isolate this species from the soil or vegetation in which *Deroceras reticulatum* was living; Warren (1932), however, did believe that he was successful in isolating a free-living form of *T. limacis*. Dobrzańska's (1958) report of this ciliate's presence in the mantle cavity of freshwater clams is interesting in this regard, but her paper does not allow the reader to know for certain if she was dealing with a genuine *T. limacis*.

In any case, there is no doubt but that both *T. limacis* and *T. rostrata* do exhibit true facultative parasitism. Their modes of entry and (less drastic) effects on their hosts set them apart from the host–parasite relationships shown by tetrahymenae belonging to other species of the genus.

The case of Tetrahymena corlissi *and vertebrate hosts.* Vertebrates less often have been involved in cases of parasitism by species of *Tetrahymena* than have invertebrates, as may be recalled from inspection of Table 1. Some of the reports in the literature are known to have been concerned with *T. pyriformis* or *T. faurei*, or else exact identity has been impossible. But, particularly in very recent years, the species *T. corlissi* Thompson, 1955, has come into prominence as a frequent parasite of aquatic vertebrates, especially amphibians and fishes. The works of Dr C. C. Speidel, most important in this area, unfortunately have appeared only in abstract form to date (Speidel, 1951–58). Dr Ruth McClung Jones and Dr Jesse C. Thompson, Jr., also are preparing papers dealing with certain aspects of the comparative morphology, systematic status, and host–parasite relationships of *T. corlissi* (personal communications).

The mode of entry generally is not known for certain. Dr Jones (see Burns *et al.* 1955; Nance, 1954; and personal communication) believes that only wounded or bruised guppies are susceptible to infection, and Speidel (1951ff.) states that poorly nourished tadpoles are attacked and successfully entered by the tissue-loving *T. corlissi*. At the time of this writing, the interesting work being carried on by Nigrelli and associates (Nigrelli, Jakowski & Padnos, 1955, 1956) at aquaria of the New York Zoological Society has not been reported in full (see abstracts by Nigrelli *et al.* 1955, 1956): they have found a variety of fishes (see Table 1), wounded or with surface lesions of some sort, susceptible to tetrahymenid ciliates

acting as 'pathogenic epibionts'. But determination of the exact identity of the species has not yet been published.

T. corlissi is very likely a bona fide facultative parasite, although more information is needed concerning many details. The very recent discovery of a strain of this species from dried moss (McArdle, 1959) is actually a little disconcerting! However, this find offers definite proof that this hymenostome ciliate is not solely parasitic, viz. an obligate parasite such as species of the trichostome genus *Balantidium*.

Miscellaneous cases. The remaining reports listed in Table 1 but not covered in the preceding paragraphs are minor in importance. They represent either accidental, unusual cases of infection of hosts by this or that species of *Tetrahymena*, or reports in which the morphological descriptions are inadequate for proper taxonomic allocation of the ciliates involved. The uncertain status of *T. parasitica*, described only from gammarids (Penard, 1922), and *T. faurei*, from a single specimen of a wounded embryonic trout (Corliss, 1953*a*), has been discussed above (see §V).

It is debatable whether any of these miscellaneous cases should be considered, in themselves, to represent true instances of facultative parasitism on the part of the tetrahymenid ciliates involved.

VII. SUMMARY

A holotrichous ciliate found repeatedly in very large numbers in the body cavity of larval midges (*Chironomus plumosus*) from Paris and Berlin areas is described as a new species of the genus *Tetrahymena*, named *T. chironomi* sp.nov. It appears to represent a true example of facultative parasitism, since parasites released from the host survive in laboratory cultures, and free-living ciliates believed identical have been isolated from fresh-water sources in nature. Over a 3-month period more than 2000 larvae, principally fourth-instar stages, were examined; about 9 % of these were found to be infected with ciliates.

T. chironomi is the thirteenth species to be added to the growing holotrich hymenostome genus *Tetrahymena*. It is taxonomically distinguishable from other members of the genus on the basis of a combination of characteristics related to its morphology and bionomics. It is one of the smallest species, has 24–27 ciliary meridians and a relatively large, spherical micronucleus, does not exhibit dimorphism or cystic stages in its life cycle, and does not possess a caudal cilium.

The new species undergoes a lethal selfing kind of conjugation, normally within the host, a short time before the larval chironomid itself succumbs from the infection. The synchronous involvement of all the ciliates in a given host in this act of sexuality suggests strongly that it is triggered in some way by the host. Whether the protozoa remain in the chironomid or are released some time before undergoing conjugation, the exconjugants invariably die.

The genus *Tetrahymena* has been growing rapidly. Eight of the thirteen species now comprising it have been implicated in reports of accidental or facultative parasitism. The taxonomic status of these species, in particular, is reviewed,

while comparing *T. chironomi* with each of them. Among the eight is the curious larval mosquito parasite discovered and described by Lamborn and Keilin in 1921. Following its rediscovery by Muspratt some 25 years later and my own recent re-examination of new material (kindly supplied by Dr Muspratt), I am able to conclude that the ciliate should be recognized as *T. stegomyiae* (Keilin, 1921) comb.nov. Its distinctive cuticular cysts aid in separating the species from congeneric forms.

Host-parasite relationships are considered in some detail, after first pointing out the difference, strictly speaking, between accidental and facultative parasitism, and noting that members of the genus *Tetrahymena* are the ciliates most frequently found exhibiting such phenomena because of their ubiquity and adaptability. Five groups of species are given special treatment with emphasis on the life cycle of the ciliate, the mode of entry into the host, and the effect on the host. All cases are considered in abbreviated form in Table 1.

T. pyriformis, the type species and the best known, has been reported a number of times within the bodies of other animals. In some cases the ciliates actually belonged to other species; in many instances the occurrences may be considered quite accidental as far as 'parasitism' is concerned. In such cases entry is probably made possible through artificial breaks or wounds in the body wall. Curiously enough, an amicronucleate *T. pyriformis* often is found with *T. chironomi* in the same specimen of larval chironomid host. Strains of *T. pyriformis* have been used the most frequently in experimental studies of facultative parasitism: inoculations into the body cavities of insect larvae, especially, have been very successful. The ciliates thrive and the hosts are killed by the infection.

T. chironomi, whose only host known to date is *C. plumosus*, causes the death of the larval chironomids after a period of days. The mode of entry remains unknown but it is postulated that it may take place through some weakened part of the body immediately following moulting. The ciliates reach a tremendous population —100,000 or 200,000 estimated in fourth-instar larvae—yet, seemingly, all to no avail, for something in the host's body fluids seems to induce total conjugation which is of a lethal selfing type. Apparently not a single exconjugant survives.

T. stegomyiae enters culicine mosquito larvae by means of a unique cuticular cyst. The infection kills the host. More data are desirable concerning the life cycle of this species in nature, but it appears to be a true facultative parasite distinguishable from all other tetrahymenids.

T. limacis and *T. rostrata* are edaphic, histophagous forms found as facultative parasites in terrestrial pulmonate gastropods. *T. rostrata* has been found free-living and in enchytraeid oligochaetes as well; it has encysted stages in its full life cycle. *T. limacis* seems to be confined to slugs and snails; it cannot form cysts and may spend only a short time outside its host. Neither species kills its host even though the ciliates may occur in fairly large numbers in the liver, renal organ, and digestive tract. Entry is probably through the mouth.

T. corlissi, one of the newer species in the genus, has been discovered in a number of vertebrates, especially amphibians and fishes. Bruises or breaks through the

skin probably serve as portals of entry. Undernourished tadpoles are very susceptible to attack by this voracious tissue-feeding ciliate. Work is in progress in several laboratories on detailed problems involving host-parasite relationships between *T. corlissi* and selected hosts.

ADDENDUM

I am indebted to the editor for allowing me to add a brief note at the time of reading proof. Three very recent papers of considerable pertinence must be mentioned, as well as two older references inadvertently overlooked. Dobrzańska (1959) has published a morphological account of tetrahymenid ciliates in several freshwater mussels, complementing her earlier work, with careful identification of two facultative parasites as *Tetrahymena limacis* and *T. pyriformis*. Kazubski (1959) has extended his host list for *T. limacis* and *T. rostrata*. Stolk (1959) has reported a '*Glaucoma*' from the central nervous system of the carp and also from amphibia: his description is brief but strongly suggests to me that the ciliate is *T. pyriformis*. It is too late to add the above data to Table 1.

The experimental work of Erhardová (1952) should be cited. This investigator used the walking-stick (*Dixippus morosus*) and several other animals, including vertebrates, as hosts, inoculating them with eight different species of ciliates and one flagellate. Although the work was not extensive, some success was obtained. Species used which without doubt are assignable to the genus *Tetrahymena* included '*Glaucoma parasiticum*', '*G. piriformis*', and '*G. vorax*'.

In one of the rare references in the literature to the taxonomically uncertain genus '*Paraglaucoma*', Hauschka & Doll (1944) briefly reported a '*Paraglaucoma* sp.' as a facultative parasite of a freshwater *Hydra* and a marine *Campanularia*. The *Hydra* invader could very likely have been *Tetrahymena rostrata*; the marine ciliate surely needs further study.

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Studies on *Penetrocephalus ganapatii*, a new genus (Cestoda: Pseudophyllidea) from the marine teleost *Saurida tumbil* (Bloch)

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INTRODUCTION

There have been many recent records (see Rao, 1959*a*) dealing with pseudophyllidean cestodes inhabiting marine teleosts in the Bay of Bengal, including the occurrence of *Bothriocephalus ganapatii* in the lizard fish *Saurida tumbil* Bl. (Rao, 1954). A remarkable feature of this tapeworm is the penetration of the intestinal wall of the host by the scolex to reach and enter the liver before undergoing final degeneration. Subhapradha (1955), whilst this work was in progress, gave an inadequate description of this cestode as *Bothriocephalus penetratus*. However, since this bothriocephalid is so unique a new genus *Penetrocephalus* is established. The reasons for the establishment of the new genus are given in this paper together with observations upon the anatomy of the above species, now named *P. ganapatii*, and a preliminary interpretation of the histochemistry of its egg-shell formation.

MATERIAL AND METHODS

The material was collected from fish caught off the coast of Waltair (Bay of Bengal), Andhra Pradesh, India, and fixed in Bouin's solution, alcoholic-Bouin fixative, or 5% formalin. Whole mounts were stained with alum carmine. For histological and histochemical examinations sections 8–12μ thick were stained according to the following techniques: (1) Heidenhain's iron haematoxylin and eosin, (2) Ehrlich's acid haematoxylin and Best's carmine, (3) Baker's acid haematein, (4) PAS-light green, (5) triple stain procedure of Himes & Moriber (1955), (6) aqueous azur I, (7) azur I-Schiff after hydrolysis in 5N HCl for 30 min. at room temperature, (8) Methasol fast blue and neutral red (Pearse, 1955). Other special procedures are mentioned later in the text.

OBSERVATIONS—ANATOMICAL

The fully developed scolex has two bothridia with smooth margins, without an apical disk, and it resembles that of *Diphyllobothrium* (Text-fig. 1). After piercing the intestinal wall the scolex attacks and penetrates the liver where it gradually degenerates and becomes invested in a cyst. It remains connected with the intra-

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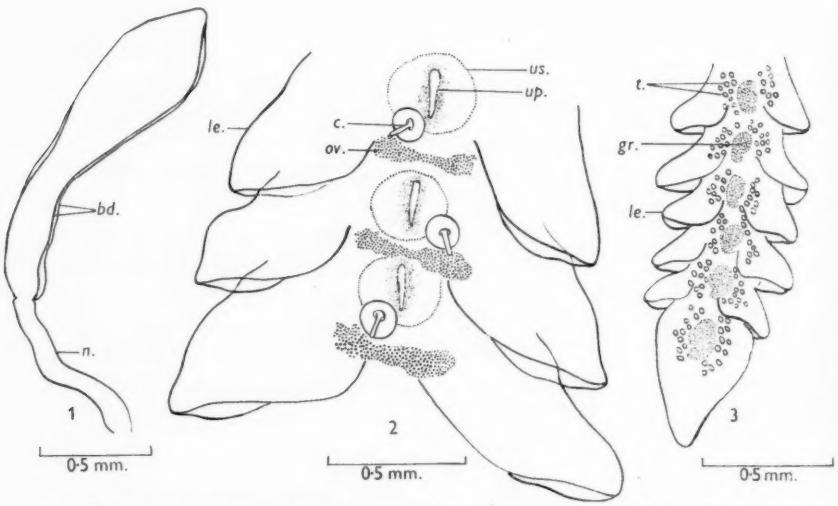
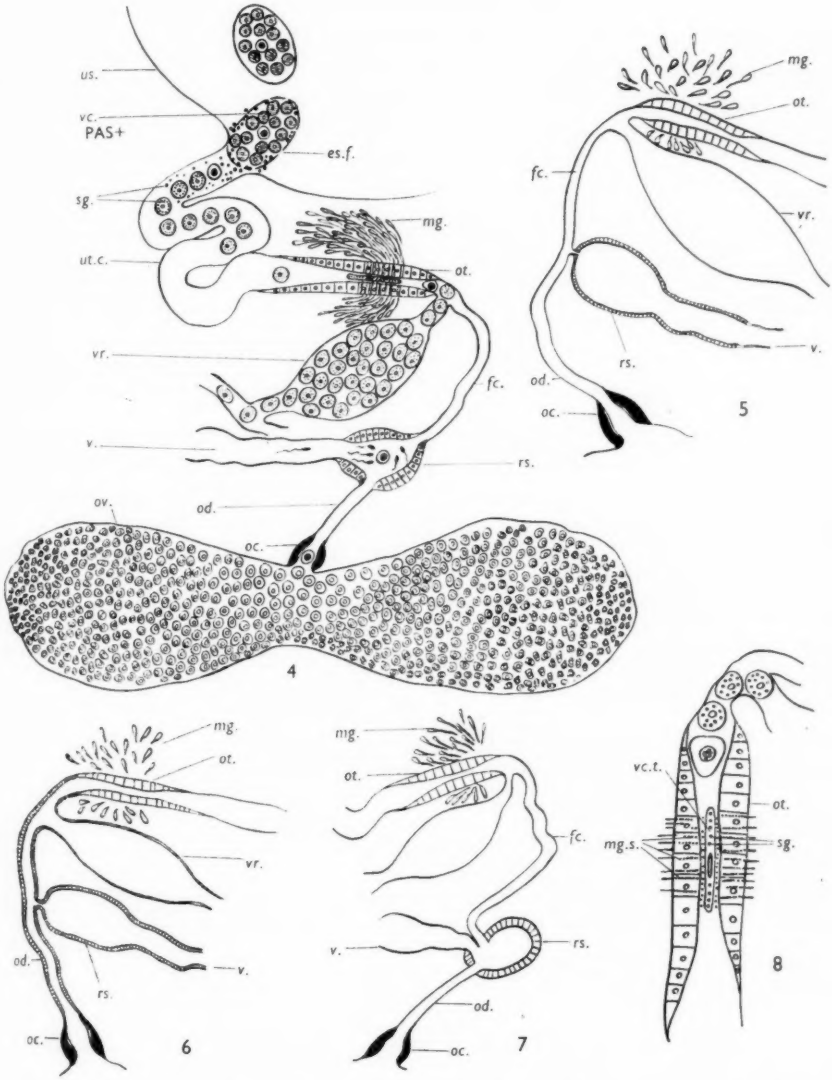
luminar strobila by the neck region lying in the body cavity. Segmentation is very prominent in the strobila. The telescoping proglottids resemble incomplete funnels with their narrower anterior regions continuous from segment to segment. Thus each proglottis presents wing-like lateral expansions (Text-figs. 2, 3). Testes vesicles are arranged in two fields which remain distinct only in the smaller, younger worms. In older worms the proglottids are relatively much thicker, each with about sixty testes showing continuous arrangement from segment to segment. The oval cirrus pouch is situated slightly either to the right or left of the mid-dorsal line opening into a genital atrium similarly placed immediately anterior to the level of the ovary. The cirrus is prominent when extruded.



Text-figs. 1–3. *Penetrocephalus ganapatii*. Text-fig. 1. Scolex. Text-fig. 2. Proglottids showing wing-like lateral expansions, uterine sac, uterine pore, ovary, and cirrus. Text-fig. 3. Terminal proglottids in a young worm.

The ovary is transversely elongated, bilobed and situated at the posterior limit of the proglottis. The ovicapt (Text-fig. 4, *oc.*) springs from its median anterior region in the transverse plane and continues as a short inclined oviduct (*od.*) opening into a small sac, the receptaculum seminis (*rs.*). The walls of the latter are distinctly cellular, and it is also joined by the broader vaginal tube (*v.*) and the narrower fertilization canal (*fc.*). Rawson (1957) called the receptaculum seminis in *Eubothrium crassum* the 'vagina sac', stating that there was a well-developed muscular layer around the cellular layer, so that the sac was contractile and forced oocytes into the fertilization canal called the 'uterine duct'. In the present cestode no evidence of a similar muscular coat is to be found. It may be argued that since the sac is in communication not only with the narrow fertilization canal but also with the relatively wider vagina, a contraction may force the oocyte into the path of least resistance, namely the vagina, since there is no valvular mechanism present. In *Schistocephalus solidus*, the receptaculum seminis

(Text-fig. 5, *rs.*) consists of a thin layer of flattened cells and there is no well-developed muscle layer; and a similar arrangement occurs in *Ligula intestinalis* (Text-fig. 6). In fact. in these tapeworms the oocyte may pass directly into the



Text-figs. 4–8. Text-fig. 4. Diagram of the female reproductive system showing scheme of egg-shell formation in *Penetrocephalus ganapatii*. Text-fig. 5. Diagram of female reproductive ducts in *Bothriocephalus scorpii*. Text-fig. 6. Diagram of female reproductive ducts in *Schistocephalus solidus*. Text-fig. 7. Diagram of female reproductive ducts in *Ligula intestinalis*. Text-fig. 8. Vitelline cell, in transit through ootype in *P. ganapatii*, being bathed in secretion of Mehlis's gland.

fertilization canal without having to enter the receptaculum seminis. In *Bothriocephalus scorpii* the thick-walled receptaculum seminis (Text-fig. 7, *rs.*) has no apparent trace of a muscle layer in contrast to the very thick muscular walls reported by Hilmy (1929). Thus Rawson's view that the receptaculum seminis is contractile does not appear to be of general significance. The vagina, after its short transverse path, turns sharply dorsalwards to open into the genital atrium close to the cirrus opening. The vitelline follicles are mostly distributed in the wing-like lateral expansions of the segment. The two transverse vitelline ducts open into a median vitelline reservoir (*vr.*) which finally opens into the ootype (*ot.*) close to the entrance of the fertilization canal. The ootype is a straight tube whose walls consist of conspicuous cubical cells. Tuft-like Mehlis's gland cells (*mg.*) surround the beginning of the ootype. The uterus, which is a continuation of the ootype, begins as a thin-walled lightly coiled tube expanding into a spherical median sac, the latter opening on the ventral side by a medial oval pore (Text-fig. 2, *up.*).

HISTOCHEMICAL OBSERVATIONS

General

Rich stores of intracellular glycogen were found in the parenchyma but it was not present in the cuticle, excretory vessels and the nerve cords, whilst the longitudinal muscles do not appear to contain any, although the immediately surrounding tissues give an intense reaction. The spermatozoa give a weakly positive reaction. Ova and vitelline cells do not contain glycogen.

Mehlis's gland and shell-globules

Mehlis's gland is strongly PAS-positive, but the reaction persists even after saliva or α-amylase treatment (Pl. 1, fig. 1). There is a positive reaction to Ehrlich's acid haematoxylin (Pl. 1, fig. 2), but Best's carmine gave a negative response. The secretion of the gland, seen in sections as minute droplets infiltrating into the lumen of the ootype through the cells in its walls, gives similar reactions. Infiltration occurs through a narrow region (Pl. 1, figs. 1, 3) at the beginning of the ootype. Beyond this narrow region, either in the ootype or in the proximal region of the uterus, no trace of the gland secretion could be detected. The shell globules in the vitelline cells, as well as the completed egg-shell, stained yellow by naphthol yellow and blue by azur-Schiff techniques but were PAS (basic fuchsin)-negative. On the other hand, the Mehlis's gland secretion is PAS-positive but naphthol yellow and azur-Schiff negative. Himes & Moriber (1955) have found that by triple staining procedure with azur-Schiff, PAS and naphthol yellow-s; DNA, vicinal hydroxy, hydroxyamino groups and basic proteins could be selectively demonstrated by blue, red and yellow colorations respectively. When this procedure was applied, the parenchymatic glycogen stained red, Mehlis's gland brilliant scarlet, the shell globules and egg-shell yellow, the nuclei blue, and the contents of the egg bluish violet. Actually, both naphthol yellow and azur I-Schiff compete with each other to stain the shell material though the latter reaction is not so well marked as in the case of the shell material in *Fasciola hepatica* (Rao, 1959*b*).

Egg-shell formation

The process of egg-shell formation in *P. ganapatii* would appear to be as follows. The oocytes pass singly into the ovicapt whose thick walls, which give a PAS-positive reaction, regulate their passage into the oviduct, to reach the receptaculum seminis. Here fertilization presumably takes place and the ovum moves towards the ootype and one or two ova with a number of vitelline cells accumulate preparatory to moving into the robust cellular ootype. The cells seem almost to be squeezed through the ootype in extremely elongated form (Pl. 1, fig. 5, *vc.t.*). During this transit they are profusely bathed in the secretion of Mehlis's gland. Once in the proximal coils of the uterus these cells regain their spherical shape as the pressure is released. An ovum at this stage is followed by a string of vitelline cells containing shell globules which are now prominent (Pl. 1, fig. 6). These vitelline cells are still PAS-negative. In the initial coils of the uterus, a little distance from the uterine sac, about 9–14 vitelline cells surround an ovum and extrude the shell material. A shell is quickly formed. The vitelline cells—now denuded of all shell material—turn PAS-positive, fast to saliva and α-amylase digestion.

Nature and functions of Mehlis's gland

Since Mehlis's gland gave positive reactions with PAS and Ehrlich's acid haematoxylin, the probable phosphatide nature of its secretion was suspected. Hence extraction with pyridine was attempted. After extraction for 24 hr. at 60° C. the gland no longer gave a PAS reaction, or at most only a weakly diffuse reaction occurred (Pl. 1, fig. 4). The sensitive acid haematein test of Baker (1946) for phospholipids could not be applied owing to lack of adequately fixed material. A mildly positive reaction was obtained, however, with acid haematein using the available material. The gland gave a positive reaction with the methasol fast blue and neutral red procedure for phospholipids (Pearse, 1955) before, but not after, pyridine extraction. Hence it may be concluded with some degree of certainty that Mehlis's gland in this cestode is elaborating and secreting a phospholipid-like substance. At present it is difficult to surmise the exact nature and functions of the secretion. Leblond, Glegg & Eidinger (1957) have affirmed '. . . no significant role can be given to lipids in the P.A.-Schiff reaction observed in paraffin sections of normal structures', and further stated that after the removal of glycogen from sections with amylase the PAS technique is specific for carbohydrate-protein complexes. In the present tapeworm the pyridine extraction test would suggest the presence of a lipid-bound material rather than carbohydrate-protein complexes. Whatever may be the precise nature of the secretion it becomes clear that the vitelline cells from the time of their origin in the follicles until their passage beyond the Mehlis's gland region of the ootype are devoid of PAS-positive substances. The strongly PAS-positive material appears much later and obviously seems to be synthesized under the influence of the Mehlis's gland secretion. It is thus tempting to suggest that the secretion—apart from its prime function of stimulating the vitelline cells to release the shell-globules—also takes part in the synthesis of yolky material. It is clear that there is no evidence for the secretion forming part of the egg-shell.

DISCUSSION

Mehlis's gland in this species is shown to be a well-developed structure surrounding the ootype and well separated from the oviduct and not just a mass of cells around the oviduct as described by Subhapradha (1955). Furthermore, the use of the term shell gland does not seem to be appropriate. Though bearing resemblance to the genus *Bothriocephalus*, the character of the scolex, the filamentous neck and the nature of the proglottids are so distinctive as shown in Table 1 that the erection of a new genus *Penetrocephalus* to accommodate this unique tapeworm, *P. ganapatii*, is justified.

Table 1. *Differences between the genera* Penetrocephalus *and* Bothriocephalus

Penetrocephalus gen.nov.	*Bothriocephalus* Rudolphi, 1808
(1) Scolex without apical disk, bothridial margins smooth, after penetrating the intestinal wall of host attacks the liver and undergoes degeneration, and finally is lodged in a cyst embedded in the liver	(1) Scolex with an apical disk, bothridial margins ruffled. Scolex intraluminar, no degeneration
(2) A slender filamentous neck lies in the body cavity of the host	(2) Neck absent
(3) Proglottids thick, resembling incomplete funnels, thus presenting conspicuous wing-like lateral expansions containing most of vitellaria	(3) Proglottids though craspedote lack the characteristic wing-like expansions

MEHLIS'S GLAND AND EGG-SHELL FORMATION

From time to time different authors have expressed diverse views on the role of Mehlis's gland in trematodes and cestodes. The most comprehensive works on the subject are those of Smyth (1956) and Smyth & Clegg (1959). It appears fairly certain that the nature and distribution of the gland are variable in different cases. In view of this fact it may be suggested that the precise functions of the secretion may also vary considerably, and even broad generalizations cannot be attempted in the existing state of our knowledge. In the present case it would appear that the gland is secreting a phosphatide, more specifically a phospholipid-like substance as is shown by the PAS, Ehrlich's acid haematoxylin, methasol fast blue–neutral red and pyridine tests. In a majority of cases the primary role appears to be associated with the release of shell globules from the vitelline cells. Thus, when the cells are bathed in the secretion it is possibly through some ionic-equilibrium phenomenon that the extrusion of shell material is manifested. The operation of such a phenomenon in cellular differentiation was recently suggested by Willmer (1958). In trematodes and cestodes where the egg-shell in many species is a quinone-tanned protein, the material for shell construction appears to come mainly if not entirely from the vitelline cells. In several instances the vitelline cells may also contain abundant quantities of food reserves such as glycogen in *Fasciola hepatica* (Stephenson, 1947; Rao, 1959*b*). In cestodes such as *Penetrocephalus ganapatii*, however, these cells appear to be merely vehicles for trans-

portation of shell precursors in the first instance, and soon after being stimulated by the secretion of Mehlis's gland, possibly imbibing some or all of it in one form or other, active synthesis of food reserves is accomplished as well as the release of shell globules. Thus, when the egg is completed, the vitelline cells lose the shell material completely but acquire nutritive material intended for the embryo. Monné (1955) said: 'It seems that the eggs or vitelline cells of most platyhelminthes and aschelminthes (nematodes, acanthocephales) are not able to elaborate any functional yolk but only the Gram-positive quinone-tanned protein spheres which can be regarded as a rudimentary yolk, undigestible and, consequently, unsuitable for the nutrition of the embryo.' This view, however, appears too generalized. More work is necessary in this direction before putting forward any concrete ideas on the nutrition of the embryo. The present work has provided a further clue as to the possible nature and yolk-synthesizing function of Mehlis's gland in this pseudophyllid. It can be said that in cases where the egg-shell is a quinone-tanned protein the secretion of Mehlis's gland triggers off the release of shell-formative globules from the vitelline cells but does not itself form part of the shell. In other cestodes such as the Cyclophyllidea its function may be quite different, as the shell in this group does not appear to be a tanned protein.

SUMMARY

1. The character of the scolex, the long filamentous neck and the morphology of the proglottids are features which justify the establishment of a new genus, *Penetrocephalus*, to accommodate the pseudophyllid species *P. ganapatii* (= *Bothriocephalus ganapatii* (Rao, 1954), *B. penetratus* (Subhapradha, 1955)) from the marine teleost *Saurida tumbil* Bloch, off Waltair, Bay of Bengal.

2. The anatomy of the female reproductive system is described in detail, and a preliminary interpretation of the histochemistry of egg-shell formation is given.

3. On the basis of histochemical tests it is suggested that Mehlis's gland, which surrounds the cellular ootype, secretes a phospholipid-like material which may aid in the release of shell precursors from the vitelline cells, but it does not form part of the shell. It appears also to be responsible for the synthesis of PAS-positive yolky matter found in these cells in the egg capsule. The functions of Mehlis's gland in helminths are discussed.

4. In a majority of helminths where the egg-shell is a quinone-tanned protein, it is considered that it is solely if not entirely derived from shell globules in the vitelline cells.

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KEY TO LETTERING

bd.	bothridia	*od.*	oviduct
c.	cirrus	*ot.*	ootype
es.f.	egg-shell formation	*ov.*	ovary
fc.	fertilization canal	*rs.*	receptaculum seminis
gr.	rudiment of female reproductive organs	*sg.*	shell globules
		t.	testes
le.	wing-like lateral expansions of proglottis	*up.*	uterine pore
		us.	uterine sac
mg.	Mehlis's gland	*ut.c.*	initial coils of uterus
mg.n.	Mehlis's gland region negative	*v.*	vagina
mg.s.	Mehlis's gland secretion	*vc.*	vitelline cell
n.	neck	*vc.t.*	vitelline cell in transit in ootype
oc.	ovicapt	*vr.*	vitelline reservoir

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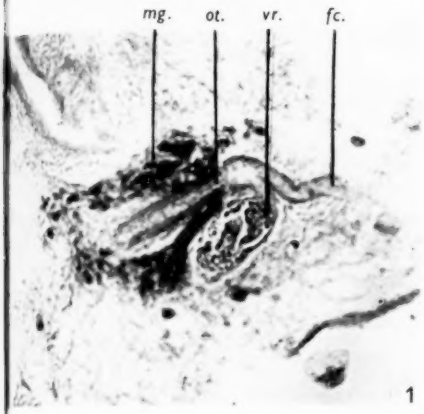

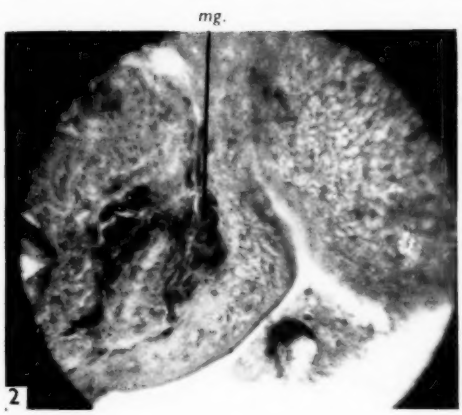

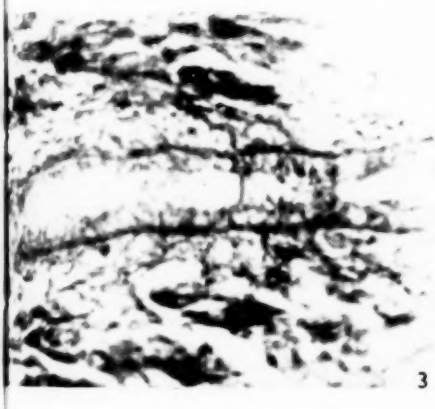

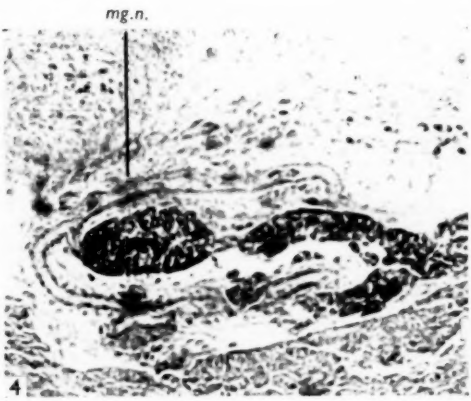

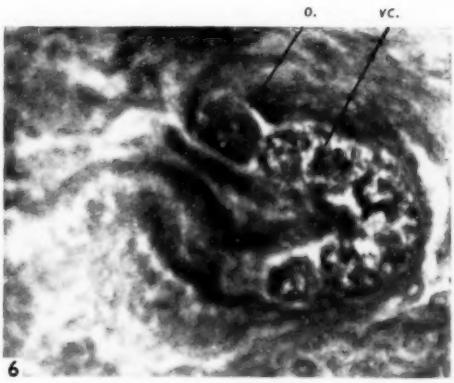

K. HANUMANTHA RAO

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EXPLANATION OF PLATE 1

Fig. 1. Section showing Mehlis's gland around ootype. (PAS-Light green after saliva digestion.)

Fig. 2. Section showing Mehlis's gland around ootype. (Ehrlich's acid haematoxylin and Best's carmine.)

Fig. 3. Section of ootype showing the secretion of Mehlis's gland infiltrating through the cellular wall. (PAS-Light green.)

Fig. 4. Transverse section of proglottis showing disposition of female reproductive ducts. Note Mehlis's gland is negative. (PAS-Light green after treatment with pyridine at 60° C. for 24 hr.)

Fig. 5. Section of ootype showing a vitelline cell in transit and being bathed by the secretion of Mehlis's gland. (PAS-Light green.)

Fig. 6. Section showing a proximal coil of uterus with ovum followed by a string of vitelline cells.

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Autoradiographic studies on *Fasciola hepatica* L.

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(*Received* 24 *July* 1959)

INTRODUCTION

In a recent contribution Gresson & Threadgold (1959) claim that the epithelial cells lining the gut of *Fasciola hepatica* pass through absorptive and secretory cycles, and that the study of electron micrographs shows that the fine processes arising from these cells are frequently, if not always, in the form of loops, both ends of which are attached to the same cell.

The present investigation was undertaken in an attempt to determine: (1) whether it is possible to differentiate by means of autoradiography between absorptive and secretory cells in the gut epithelium of the liver fluke; (2) the function of the protoplasmic processes of the epithelial cells; and (3) the metabolic fate of the excess iron that the parasite must collect as it feeds on the blood and liver tissue of its host (Grembergen, 1950).

MATERIALS AND METHODS

Isotopes used. (1) Iron-59, obtained from the Radiochemical Centre, Amersham, as a solution of ferric chloride in 0·1 N hydrochloric acid. Before use, an appropriate amount of a 0·1 % solution of disodium citrate was added so as to make the solution into a Sörensen buffer of pH 4·0 the purpose being to reduce the acidity of the solution without causing precipitation of ferric hydroxide. (2) L-Phenylalanine–C-14, obtained from the same source.

Procedure. Living flukes were removed from cow and sheep livers (obtained from the Belfast abattoir) and immediately washed in the saline solution recommended by Stephenson (1947). In Expt. 1 a drop of the radioactive iron solution was injected through the mouth and into the gut by means of a fine micropipette manipulated by hand under a binocular microscope. Under sufficient pressure the solution flowed into the crura and their diverticula almost throughout the entire body. The success of an injection could be easily ascertained by adding to the solution a drop of Evans blue. In Expt. 2 heparinized mouse blood was injected into the gut of the parasites. This blood was labelled by the injection into mice of four equal daily doses of iron solution to a total activity of approximately 30 μc. and 15 μg. of total iron element. Blood was collected 3 days after the last injection. In Expt. 3 a solution in saline of L-phenylalanine–C-14, was employed.

Maintenance and fixation of injected parasites. Some flukes were placed in beakers containing Stephenson's saline and kept in an incubator at 37° C. Flukes were removed and fixed at intervals of 5, 15, 30 and 60 min.

Stephenson (1947), taking suitable precautions, has kept flukes alive in a special saline for periods of up to 60 hr. It is very doubtful, however, whether after some hours *in vitro* the animals are still healthy and normal and free from bacterial attack. This is, of course, one of the difficulties associated with metabolic studies on many internal parasites. We feel that we have overcome this difficulty by inserting freshly collected flukes into the abdominal cavity of mice, where they feed (usually on the peritoneum) and remain active for at least 1 week (longer periods were not tested). By applying this procedure we were able to recover living flukes from their temporary hosts at periods varying from 6 hr. to 5 days from the time of injection. The introduction into the temporary host was made under anaesthesia (Nembutal) through a small incision in the body wall. The worms were recovered after the hosts were killed.

The fixatives used were Carnoy's acetic alcohol (with methyl alcohol in place of ethyl alcohol) and Flemming's fluid. Sections were cut at 7·5μ in thickness. The autoradiographs, after development and fixation, were stained in methyl green pyronin.

Autoradiography. The stripping film technique of Doniach & Pelc (1950) was used. The slides were covered with Kodak AR 10 film and exposed for 8 days in the case of iron, and 15 days in the case of carbon.

RESULTS

Experiment 1 (iron in solution). Irrespective of the interval between injection and fixation in Flemming's solution, a high concentration of autoradiographic grains appear in all the cells of the gut epithelium. We could not, however, establish any clear quantitative difference between cells in the columnar, pyramidal and squamous conditions. Activity was not observed in the many protoplasmic processes originating from the epithelial cells. Specimens fixed 2 hr. or longer subsequent to injection (Flemming's solution), in contrast to those fixed at shorter intervals, show an equally high degree of activity in the intestinal and the cuticular epithelia, but autoradiographic grains are absent from all other tissues. Flukes fixed in Carnoy's fluid, however, exhibit high activity in the myoblasts and in the cells forming the walls of the excretory tubules as well as in those of the intestinal and cuticular epithelia.

Experiment 2 (iron-labelled blood). Owing to the procedure adopted for labelling blood it is impossible to determine, without further work, whether or not the isotope is present as free iron in the plasma. The autoradiographic picture is similar to that obtained in Expt. 1, except that, even with Flemming's fixative, the cuticular epithelium always shows heavy labelling (Pl. 1, figs. 3, 4, 6 and 7).

Experiment 3 (phenylalanine in saline). Specimens fixed in Flemming do not exhibit activity in any of their tissues. Those fixed in Carnoy 15 min. after injection show high activity in the cuticular epithelium, the myoblasts and the cells of the excretory canals, but not in the intestinal epithelium. The picture is similar for flukes fixed at later periods up to 24 hr. (Pl. 1, figs. 2, 5). Specimens fixed 5 min. after injection show no activity.

In autoradiographs of specimens left in temporary hosts for 5 days before removal and fixation, the tracer is concentrated in the cuticular epithelium, and to a less extent in the myoblasts, but is not present in the cells of the excretory tubules.

DISCUSSION

There are striking contrasts between the activity distribution in autoradiographs of the tissues of *F. hepatica* when the fixatives are varied. From this we draw the following conclusions, and our results should be considered with these in mind: (1) the picture obtained with each fixative is only a partial one, and (2) a wide range of fixatives should be used in future work so that conclusions may be drawn as to the nature, as well as the position, of the labelled compounds in fixed tissues. For example, the presence of iron in the gut and the cuticular epithelia fixed in Flemming's fluid supports the hypothesis that the iron in these tissues is bound to lipids or lipoproteins. The high activity of the myoblasts and the cells of the excretory tubules of flukes preserved in Carnoy indicates that the iron is bound to proteins. Flemming's fixative preserves lipids while Carnoy's fluid fixes proteins, although not *all* proteins (Baker, 1958).

With both iron-labelled blood and free iron, a high density of radiographic grains appears in the tall (columnar and pyramidal) and the short (squamous) cells of the intestinal epithelium. Our experiments, therefore, confirm the earlier conclusions of Gresson & Threadgold (1959), based on the examination of histological preparations and electron micrographs, that both tall and short cells are absorptive in nature. Gresson & Threadgold also claim that the short cells are transformed into either absorptive or glandular cells, and that the epithelium passes through absorptive and secretory cycles. In general, these conclusions agree with those of Müller (1923). The presence of secretion in cells of the intestinal epithelium of *F. hepatica* was observed in Gresson & Threadgold's histological preparations, but gland cells were not shown in their electron micrographs. Gland cells, they believe, are not present in parts of the alimentary canal containing food. They conclude, therefore, that the discharge of secretion to the lumen of the gut and the transformation of gland cells to the absorptive phase must take place very rapidly.

In our experiments the protoplasmic processes of the epithelial cells do not appear to be labelled. Gresson & Threadgold, however, consider that these processes decrease in number during the later stages of the accumulation of secretion within a gland cell, and suggest that they may be concerned with the absorption of nutriment from the cavity of the gut. The processes are extremely fine and their exact arrangement and morphology cannot be determined with light microscopy. Futher autoradiographic experiments may reveal their function.

The absence of labelling from the gut cells when phenylalanine is injected is not surprising, for the amino acid absorbed is probably passed through these cells fairly rapidly so that its concentration does not build up.

The high concentration of both iron and amino acid in the cells described by Alvarado (1951) as *myoblasts*, and by other authors as *gland cells*, is interesting. If these cells are myoblasts that produce muscle fibres and remain in physical

communication with them, as Alvarado argues, the presence of both these isotopes might be connected with the formation of myoglobin. Myoglobin, or myoglobin-like protein, has been detected in *F. hepatica* by Grembergen (1950). We have observed labelled prolongations of these cells extending between the layers of muscle fibres but could not detect cell processes that enter the cuticular epithelium, as claimed by some authors (see discussion in Alvarado's paper).

The high degree of activity (iron and phenylalanine) in the cuticular epithelium provides evidence in support of Alvarado's designation of this layer as a cuticular epithelium. A structure that accumulates rapidly and incorporates metabolites cannot be a dead cuticle, as believed by previous authors, but must be a metabolizing tissue. Further, in favourable sections of our material, nuclei are visible within the epithelium. We consider that this epithelium is either excretory or secretory in function, serving for the elimination of excess metabolites, or alternatively for the secretion of mucus on to the surface of the body. It is also conceivable that iron accumulates passively in the cuticular epithelium and remains there as an insoluble deposit. It was in the hope of gaining some information concerning the function of this tissue that we left injected flukes in temporary hosts for 5 days prior to fixation. That the relative tracer content of the cuticular epithelium increases during this period, indicates that part of the tracer utilized in the myoblasts is later transferred to the cuticular epithelium. This seems to apply to both amino acids and iron. It may be tentatively concluded, therefore, that, whilst speedy excretion certainly takes place through the excretory system proper, relatively large amounts of excess organic substances and of iron accumulate in the myoblasts and the cuticular epithelium, and are eliminated from the latter in the mucus.

SUMMARY

Iron-59 (as a ferric chloride solution or as a component of haemoglobin) was introduced into the lumen of the gut of *Fasciola hepatica*. It was detected by autoradiography in high concentrations in the intestinal epithelium, the cuticular epithelium, the myoblasts and the cells forming the walls of the excretory tubules. Carbon-14, introduced as L-phenylalanine–C-14, was concentrated in the same tissues, with the exception of the intestinal epithelium. These findings are discussed in relation to the function of the respective tissues.

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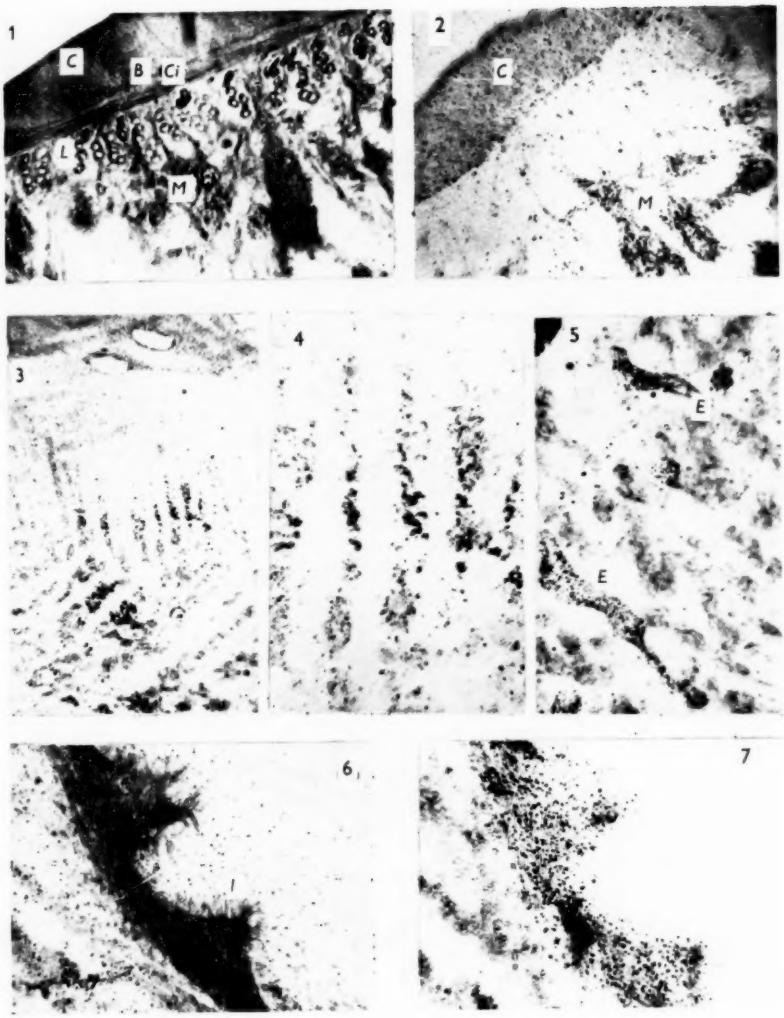
1
C
B
Ci
L
M
2
C
M
3
4
5
E
E
6
7

EXPLANATION OF PLATE 1

Fig. 1. Transverse section of part of body to show: cuticular epithelium (*C*), basal membrane (*B*), layer of circular muscles (*Ci*), bundles of longitudinal muscles alternating with the fine processes of the myoblasts (*L*), myoblasts (*M*). Flemming's fixative. Methyl green pyronin. × 290.

Fig. 2. Autoradiograph. Specimen given carbon-labelled phenylalanine. Carnoy. Fixed 24 hr. after injection. Cuticular epithelium (*C*) active. Myoblasts (*M*) with high concentration of activity. Note that fine processes of myoblasts are active, whilst longitudinal muscle bundles are inactive. × 1357.

Fig. 3. Autoradiograph. Specimen given iron-59. Flemming. Fixed 30 min. after injection. The section extends towards the middle region of the body where, in addition to longitudinal muscles, diagonal muscles are present. The fine processes of the myoblasts are active and extend between the muscle bundles towards the cuticular epithelium. × 542.

Fig. 4. Autoradiograph. Same section as fig. 3. To show more clearly activity in fine processes of myoblasts. × 1703.

Fig. 5. Autoradiograph. Specimen given carbon-labelled phenylalanine. Carnoy. Fixed 2 hr. after injection. Excretory tubules (*E*). *Note.* The cells forming the walls show activity, but as the tubules are cut tangentially, the photograph gives the impression that the autoradiographic grains are in the lumen of the tubules. × 79.

Fig. 6. Autoradiograph. Specimen given iron-labelled blood. Flemming. Fixed 6 hr. after injection. To show two short pyramidal cells of the intestinal epithelium and their processes (*l*). The cell boundaries are indistinct and the nuclei are not well shown at this focus. × 1703.

Fig. 7. Autoradiograph. Same cells as in fig. 6. Autoradiographic grains in focus. The grains are emphasized by over-exposure during printing. × 1703.

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Experiments on the encystation of *Opalina* in *Rana temporaria*

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CONTENTS

I. Introduction 171
II. Material and methods 172
III. Results 173
IV. Discussion 177
V. Summary 180
References 180

I. INTRODUCTION

The family Opalinidae comprises bi- and multinucleate, ciliated protozoa parasitic in the gut of cold-blooded vertebrates; most species are harmless commensals of amphibia. In general features, the life cycles of all opalinids parasitizing amphibia appear to be identical (see Metcalf, 1909, 1923, 1940, for comprehensive and critical reviews of the literature). The adult opalinids live in the recta of frogs and toads, and, during most of the year, multiply asexually by binary fission. In spring, as the reproductive season of the host approaches, the multiplication rate of most of the opalinids increases so that growth no longer keeps pace with division; progressively smaller forms are produced, and these minute forms eventually encyst. A few individuals which do not decrease in size do not encyst, but remain in the rectum of the host, presumably maintaining the infection there. The opalinid cysts are passed in the faeces of the host, and after being ingested by tadpoles, hatch in the intestine, and eventually give rise to gametes. Fusion of gametes then occurs, and the products of sexual reproduction ultimately give rise to the adult opalinids which persist in the adult frog or toad.

Although Metcalf (1909) showed that infections of opalinids can be produced experimentally, by feeding either cysts or adults to tadpoles and adult frogs, infection with opalinids is probably normally achieved by the ingestion of cysts by tadpoles. Under natural conditions, it is unlikely that the adult opalinids survive long enough outside the body of the frog to infect new hosts, and, as Metcalf points out, the feeding habits of adult frogs are such that infection of adults by cysts in water must be very infrequent, whereas oral ingestion of cysts by tadpoles must be relatively common.

The seasonal reproductive activity of amphibia implies that tadpoles are available for infection with opalinids only during a relatively short period of each

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year. Opalinid cysts are produced in adult amphibia only during the breeding season of the host, an observation which has been confirmed for several species of Opalinidae. This limitation of the production of infective stages of the parasite to the time of year when potential new hosts are available suggests a remarkable adaptation by the parasite to the habits of its host.

The mechanism whereby the life cycle of opalinids is correlated with that of the host is of considerable interest. The seasonal production of cysts could be due to inherent cyclical changes in the opalinids, or to stimuli from the external environment or from the host. Experimental work suggests that the stimulating factors are produced by the host. Bieniarz (1950) reported that opalinid cysts appeared in male *Rana esculenta* only during the frog's breeding season, or after the frogs were injected with human pregnancy urine. He concluded that the encystation of the protozoa was influenced, directly or indirectly, by gonadotrophic hormone in pregnancy urine, or by gonadotrophins produced by the sexually mature frog. Čehović (1956) described experiments in which *R. esculenta* and *R. temporaria* were injected with pregnancy urine, chorionic gonadotrophin or frog pituitaries. Opalinid cysts were found in frogs treated in January or February, whereas cysts did not appear in untreated frogs until March. Treated frogs apparently showed a premature development of their reproductive organs, but the doses of hormones which induced encystation of the opalinids did not always provoke full sexual maturity of the host. Čehović concluded that the correlation between the life cycles of parasite and host was due to the seasonal activity of the pituitary of the host, probably to the production of gonadotrophin.

Unfortunately, neither of these workers gave any details of the conditions in which their experimental and control frogs were kept, so the possibility remains that the encystation of the opalinids might have been influenced by environmental changes, rather than by the injections of gonadotrophins. Their work indicates, however, that the encystation of opalinids is not dependent on a seasonal rhythm in the protozoa, and suggests that it is closely correlated with reproductive changes in the host. Confirmation of this correlation was therefore sought.

The work to be described is little more than a series of preliminary experiments; the results obtained, however, suggest possible lines for future research, and are therefore reported, as they may be of interest to other workers concerned with the relationships between amphibia and their parasites.

II. MATERIAL AND METHODS

The observations were made on natural infections of *Opalina* in adult male and female *Rana temporaria*. A detailed morphological study of the opalinids was not made, but in general features they resembled *Opalina ranarum*.

Frogs were obtained from a dealer in four batches; one in September, 1956, and three between September and November, 1957. These frogs were placed in glass tanks in an unheated, poorly lit shed, and were thus exposed to the normal seasonal changes in temperature, but not to marked changes in light intensity. A constant supply of water was available to the frogs in bowls of weak saline

(0·15 % NaCl in tap water); saline was used in preference to tap water, as a prophylactic measure against the bacterial disease 'Red Leg'. Frogs to be used in experiments were removed from the stock tanks at the beginning of each experiment, and isolated, singly or in groups of two, in bell-jars kept in the laboratory. These jars were screened from direct light by thick cloths, and each contained a few stones in a shallow layer of 0·15 % NaCl. Food was never supplied to either untreated or experimental frogs.

Substances were administered to the experimental frogs by injection into the dorsal lymph sac, as described by Hobson (1952). Mammalian chorionic gonadotrophin (Pregnyl, manufactured by Organon Laboratories Ltd.) was dissolved in 0·75 or 0·9 % NaCl, and given in daily doses of 1 ml., each containing 20–50 i.u. of the hormone. Frog pituitaries, removed from frogs recently killed by a blow on the head, were teased in 0·75 or 0·9 % NaCl, and injected as a suspension of the glands in 1 ml. of saline; the pituitaries were injected either in one dose at the beginning of the experiment, or in several doses at intervals throughout the experimental period. In each experiment, additional frogs, also kept in the laboratory, were injected with an equivalent number of 1 ml. doses of 0·75 or 0·9 % NaCl.

At the end of an experiment, the cloacal fluid of male frogs was examined for spermatozoa. Each frog was then killed by a blow on the head, and portions of the reproductive organs were placed in Bouin's solution for subsequent histological examination of sections stained with Mayer's haemalum and eosin. The rectal contents of each frog were examined microscopically for the presence of adult opalinids and cysts.

At intervals throughout the experimental period, frogs were removed from the stock tanks in the shed, killed, and examined in the same way as the experimental frogs; these animals are subsequently referred to as 'untreated' frogs.

III. RESULTS

A few of the frogs examined were not infected with *Opalina*; these animals are not included in the present data. The results obtained from all the experiments are given in Tables 1–4. Details of the temperatures to which individual untreated frogs were exposed before being killed are not included in Table 1. A few of the frogs included in this group were killed as soon as they were received from the dealer, and the temperatures to which these animals had been exposed were not known. Those untreated frogs which had been kept in the unheated shed, however, were exposed to much greater daily fluctuations in temperature than those kept in the laboratory, with a gradual decline in temperature from October to December; the maximum and minimum temperatures recorded in the shed throughout these months were 19 and 1° C. Apart from differences in environmental temperature and experimental treatments, the group of untreated frogs differs from the other groups in two respects; no frogs were examined in September, and more females were examined than males, instead of approximately equal numbers of each sex. It is unlikely, however, that these differences influence the fact that *Opalina* cysts

were found in only one of the untreated frogs (Table 1), and it is noteworthy that in this frog only a very few cysts were found.

None of the frogs kept in the laboratory and injected with saline became sexually mature, but a higher proportion of these frogs contained *Opalina* cysts than did the untreated frogs (Table 2). Pregnyl, in the doses used, also had no discernible effect on the reproductive organs of any of the frogs; the relative insensitivity of *Rana temporaria* to chorionic gonadotrophin has been observed by other workers

Table 1. *The formation of* Opalina *cysts in untreated* Rana temporaria *exposed to normal seasonal changes of temperature*

No. of frogs	Sex of frogs	Month	Sperm in cloacal fluid, or eggs in oviducts*	*Opalina* cysts*
1	♀	Oct.	0	+
6	♀	Oct.	0	0
3	♂	Nov.	0	0
4	♀	Nov.	0	0
4	♀	Dec.	0	0

* + = present; 0 = absent.

	♂	♀
Total no. of frogs with *Opalina* cysts	0	1
Total no. of frogs without *Opalina* cysts	3	14

Table 2. *The effect of saline* (0·75 *or* 0·9 %) *on the formation of* Opalina *cysts in* Rana temporaria

No. of frogs	Sex of frogs	Month	Duration of experiment (days)	Temperature range during experiment (° C.)	No. of 1 ml. injections	Sperm in cloacal fluid, or eggs in oviducts*	*Opalina* cysts*
1	♂	Sept.	4	16–23	1	0	0
1	♂	Sept.	7	15–23	6	0	0
1	♀	Sept.	7	15–23	6	0	+
1	♀	Sept.	5	16–20	1	0	0
1	♀	Sept.	5	16–20	4	0	0
3	♂	Oct.	6	13–23	4	0	0
1	♂	Oct.	5	17–22	1	0	0
1	♀	Oct.	5	17–22	4	0	0
1	♂	Oct.	5	16–24	1	0	0
1	♀	Oct.	8	16–24	2	0	+
1	♂	Oct.	6	13–23	4	0	+
1	♀	Oct.	7	17–21	1	0	0
1	♂	Nov.	8	12–23	1	0	+
1	♀	Nov.	11	13–24	2	0	+
1	♂	Dec.	10	11–21	1	0	0
1	♀	Dec.	8	11–21	1	0	0

* + = present; 0 = absent.

	♂	♀
Total no. of frogs with *Opalina* cysts	2	3
Total no. of frogs without *Opalina* cysts	8	5

(van Oordt, Beenakkers, van Oordt & Stadhouders, 1954). The two frogs given the largest dose (300 i.u.) both contained cysts; on the other hand, cysts were present in a frog which was given only 20 i.u., but not in any of the frogs treated with 200 i.u. of the hormone. There was thus no evidence that the opalinids showed a graded response to chorionic gonadotrophin (Table 3). Since Pregnyl appeared to have the same effect as saline on both the frogs and their opalinids, its use was discontinued, and no data were obtained for the effects of Pregnyl in November or December.

Table 3. *The effect of chorionic gonadotrophin (Pregnyl) on the formation of* Opalina *cysts in* Rana temporaria

No. of frogs	Sex of frogs	Month	Duration of experiment (days)	Temperature range during experiment (° C.)	Total dosage (i.u.)	Sperm in cloacal fluid, or eggs in oviducts*	*Opalina* cysts*
1	♂	Sept.	7	15–23	300	0	+
1	♀	Sept.	7	15–23	300	0	+
3	♀	Sept.	5	16–20	80	0	0
1	♀	Sept.	5	16–20	80	0	+
4	♂	Oct.	6	13–23	200	0	0
1	♂	Oct.	4	20–22	20	0	0
1	♀	Oct.	2	17–21	20	0	+
1	♀	Oct.	2	17–22	20	0	0
1	♀	Oct.	5	17–22	65	0	0

* + = present; 0 = absent.

	♂	♀
Total no. of frogs with *Opalina* cysts	1	3
Total no. of frogs without *Opalina* cysts	5	5

All the male frogs responded to the pituitary injections by passing sperm, and *Opalina* cysts were present in the majority of these frogs. Only one of the females was visibly affected by treatment with pituitaries, and this was the only female in which cysts were found (Table 4).

The method of recording cysts as merely present or absent gives no indication of the numbers of cysts present. It was not considered feasible to count the relative numbers of cysts and adult opalinids present; variations in the nature of the rectal contents would have rendered the consistent preparation of even suspensions of opalinids very difficult, and, in addition, the total opalinid population appeared to be influenced by the character of the rectal contents. Cysts were exceptionally sparse, however, in four frogs—in the one untreated frog which contained cysts, in one injected with saline, and in two injected with Pregnyl.

The results obtained from all the groups of frogs are summarized in Table 5. Compared with encystation in untreated frogs, the formation of *Opalina* cysts was stimulated equally in frogs injected with saline or Pregnyl, and was further enhanced in frogs injected with pituitaries.

The testes of all the male frogs killed during September to December were

examined histologically. These frogs include some not accounted for in the previous data, as they were injected with saline or Pregnyl, but kept in the unheated shed instead of in the laboratory. Table 6 shows the distribution of *Opalina* cysts in frogs whose testes have been grouped into three categories, according to those

Table 4. *The effect of frog pituitaries on the formation of* Opalina *cysts in* Rana temporaria

No. of frogs	Sex of frogs	Month	Duration of experiment (days)	Temperature range during experiment (° C.)	No. of pituitaries injected	Sperm in cloacal fluid, or eggs in oviducts*	*Opalina* cysts*
1	♂	Sept.	4	16–23	7	+	+
1	♀	Sept.	5	16–20	4	0	0
1	♂	Oct.	8	20–22	4	+	+
1	♂	Oct.	5	17–22	4	+	0
1	♀	Oct.	7	17–21	3	0	0
1	♂	Oct.	5	16–24	4	+	0
1	♀	Oct.	6	16–23	8	0	0
1	♀	Oct.	8	16–24	6	0	0
1	♀	Oct.	20	19–23	4	0	0
1	♂	Nov.	9	13–24	4	+	+
1	♀	Nov.	11	13–24	6	+	+
1	♀	Nov.	4	18–23	3	0	0
1	♂	Nov.	8	12–23	2	+	+
1	♂	Dec.	10	11–21	2	+	+

* + = present; 0 = absent.

	♂	♀
Total no. of frogs with *Opalina* cysts	5	1
Total no. of frogs without *Opalina* cysts	2	6

Table 5. *The effect of different treatments on the formation of* Opalina *cysts in* Rana temporaria

	Total no. of frogs	% of frogs with *Opalina* cysts
Untreated frogs*	18	6
Frogs injected with saline†	18	28
Frogs injected with chorionic gonadotrophin†	14	29
Frogs injected with frog pituitaries†	14	43

* Exposed to normal seasonal changes of temperature.
† Kept at room temperature.

Table 6. *The condition of the testes in* Rana temporaria *infected with* Opalina

	Condition of testes		
	Spermato-genesis	Resting	Spermiation
No. of frogs with *Opalina* cysts	0	2 (25%)	6 (75%)
No. of frogs without *Opalina* cysts	13 (50%)	10 (38%)	3 (12%)

described by van Oordt (1956): spermatogenesis, indicated by the presence of secondary spermatogonia, spermatocytes or spermatids; resting, in which there was no sign of spermatogenesis or spermiation, and in which all the testis tubules were filled with sperm bundles; and spermiation, evidenced by the presence of spermatozoa in the cloacal fluid, or by empty tubules from which the spermatozoa had been discharged. *Opalina* cysts were present in only eight out of the thirty-four frogs examined; it is of interest, however, that cysts were never found in frogs in which active spermatogenesis was occurring, and that most of the frogs with cysts were also discharging sperm.

IV. DISCUSSION

The present results confirm those of Bieniarz and Čehović, that the seasonal production of opalinid cysts is not due to cyclical changes inherent in the protozoa; the formation of *Opalina* cysts was stimulated in all the groups of treated frogs, at a time of year when cysts are not normally produced.

Before considering the factors which may influence the formation of opalinid cysts, it is relevant to consider briefly the changes normally occurring in the amphibian environment at the breeding season, the mechanism whereby amphibian reproductive cycles are controlled, and some of the seasonal physiological changes which occur in amphibia.

Amphibia emerging from hibernation at the end of winter are exposed to increasing intensities and duration of light, and to higher temperatures; in addition, they start feeding, after several months without food. In early spring, the adults become sexually mature, and it is well established that the condition of amphibian reproductive organs is controlled by gonadotrophins secreted by the pituitary; the output of gonadotrophins increases in response to external stimuli (Bullough, 1951; Smith, 1955; van Oordt, 1956), and temperature is the most important factor controlling the activity of the pituitary in some species of amphibia. Van Oordt (1956) has reviewed the factors affecting the spermatogenetic cycle of *R. temporaria*. Light and nutrition are not important, but van Oordt (1956, 1956*a*) found that both spermatogenesis and spermiation are affected by temperature; for instance, spermiation was induced in frogs kept at 21–23° C. during November and December, whereas, in the same months, sperm were not passed by frogs kept at 5–12° C. Comparable data for the effects of temperature on ovulation in *R. temporaria* have not been found, but, in natural conditions, the mean spawning date of this species is influenced by the rainfall and temperature in the pre-spawning period (Savage, 1935), and experimental changes in temperature affected ovulation in *Bufo arenarum* and *Rana pipiens* (Smith, 1955). In addition to stimulating the maturation of the testes and ovaries, gonadotrophins also induce the production of hormones by the gonads; in amphibia, these gonadal hormones control the development of the secondary sexual characteristics (Smith, 1955). Sexual maturity in amphibia is thus preceded and accompanied by marked increases in the production of gonadotrophins and gonadal hormones.

In addition to these hormonal changes, the reproductive cycle in amphibia is accompanied by many other physiological changes. The emergence of amphibia

from hibernation implies a general increase in metabolic rate, together with an increase in the level of nutrition. Seasonal changes have been recorded in the levels of liver glycogen and blood sugar in *R. temporaria* (Smith, 1950), and in the serum levels of calcium and potassium in *Xenopus laevis* (Zwarenstein, 1933; Shapiro & Zwarenstein, 1933; Zwarenstein & Shapiro, 1933). Doubtless these are only a few of the physiological changes occurring in amphibia with the onset of spring.

The possibility must be considered that factors in the external environment directly influence the formation of opalinid cysts. On the basis of the present experiments, this possibility cannot be eliminated entirely; the enhanced encystation observed in the groups of treated frogs, compared with that in untreated frogs, may have been due to the higher temperature of the laboratory. However, encystation was not enhanced to the same degree in all the groups of treated frogs; cysts were found in a higher proportion of frogs injected with pituitaries than in those injected with saline or Pregnyl. Since all the treated frogs were kept under similar conditions, the external environment was not the only factor affecting encystation; additional stimulating factors were provided by injecting pituitaries into the host frogs.

It is suggested, however, that the increased temperature of the laboratory affected *Opalina* indirectly, rather than directly, by inducing changes in the host. The increase in temperature would have affected the development of the gonads of the host frogs, but to a lesser degree than the injections of pituitaries. Thus the enhancement of encystation in frogs injected with saline or Pregnyl may have resulted from the premature development of the gonads, even though none of these frogs became sexually mature. In the frogs injected with pituitaries, encystation was always correlated with sexual maturity. Additional confirmation of a correlation between encystation and the sexual development of the host was supplied by the observation that *Opalina* cysts were most commonly found in frogs whose testes had developed to the condition normally occurring at the breeding season. The results of the present work thus confirm the conclusions of Bieniarz and Čehović, that the seasonal production of opalinid cysts is in some way correlated with the development of the gonads of the host; as found by Čehović, encystation is not dependent on the full sexual maturity of the host, although, in the present experiments, cysts were more commonly found in sexually mature than immature frogs.

It is not possible, at present, to decide which of several possible factors induce cyst formation in opalinids. Some of the results obtained, however, seem to indicate which factors are most likely to be concerned, though final proof will probably depend on *in vitro* work using cultures of the protozoa.

It seems improbable that the behaviour of *Opalina* was influenced directly by gonadotrophins. Chorionic gonadotrophin (Pregnyl) had no effect on cyst formation. An accurate assessment of the amount of frog gonadotrophins injected cannot be obtained from the numbers of pituitaries injected, as individual pituitaries may vary considerably in their gonadotrophin activity. In contrast to the male frogs injected with pituitaries, only one of the females became sexually mature, but this may be due to the fact that the ovary is less sensitive to gonadotro-

phins than the testis. Since the females received as many, or even more, pituitaries than the males, it is unlikely that they received markedly lower doses of gonadotrophins than the males. Even so, cysts were found in only one female frog, whereas the majority of the male frogs contained cysts. If gonadotrophins stimulate the formation of *Opalina* cysts directly, it is difficult to understand why cysts were not found in more of the female frogs injected with frog pituitaries.

It seems, therefore, that some factor or factors other than gonadotrophins are concerned in provoking the seasonal production of opalinid cysts. The increased nutritional state of the host at the breeding season is obviously not important, as the frogs used in the present work were not fed at any time after they were received from the dealer. Any of the other physiological changes previously mentioned may be significant, but it is suggested that the formation of cysts by *Opalina* may be induced by the gonadal hormones of the host. The output of these hormones increases just before and during the breeding season, and presumably the amount of hormone produced varies in different individuals; if gonadal hormones are indeed concerned in the encystation of *Opalina*, differences in the amounts produced in individual frogs may explain why cysts were not always found in male frogs which appeared to be sexually mature, as they were passing sperm. In addition, the levels of gonadal hormones in the frogs which were not sexually mature, but in which cysts were present, may have been sufficiently high to influence the opalinids. Little is known about amphibian gonadal hormones, but those of mammals are noted for their mitogenetic effect; oestrogens and androgens stimulate cell division in various mammalian organs and tissues, some tissues being more sensitive to the hormones than others (Bullough, 1946, 1955). A stimulus to cell division is a prerequisite for the formation of opalinid cysts, as cysts are formed only after a period of intense cell division, by which minute individuals are produced from adult forms. It is known, too, that in some mammals both androgens and oestrogens may be excreted by the liver into the intestine, and these hormones have been identified in mammalian faeces (Burrows, 1949). If the gonadal hormones of amphibia resemble those of mammals, then they fulfil the requirements of the factor which stimulates opalinids to encyst: their production increases at the breeding season of the host, they stimulate cell division, and they are present in the rectum where the opalinids are found. It is conceivable that the synchronization of the production of opalinid cysts with the breeding season of the host has evolved as the result of these parasites developing a high degree of sensitivity to the gonadal hormones of their hosts.

The life cycle of another parasite of *R. temporaria* has also been shown, by observation and experiment, to be correlated with the reproductive cycle of the host. The trematode *Polystoma integerrimum*, parasitic in the bladder of *Rana temporaria*, lays its eggs only when the host frogs are spawning; the larva emerging from the egg becomes attached to the gills of a tadpole, and migrates to the bladder when the tadpole metamorphoses to the adult (Gallien, 1935). Experiments have shown that this correlation between the life cycles of parasite and host is not fortuitous (Miretski, 1951); frogs were induced to spawn prematurely, by injections

of frog pituitaries, and *Polystoma* in these frogs, unlike *Polystoma* in untreated, sexually immature frogs, produced fertilized eggs. The similarities, throughout the year, in the histological condition of the reproductive organs of *Polystoma* and its host are so striking that Gallien suggested that the trematode may be directly influenced by the hormones of its host. As with opalinids, the seasonal changes in the life cycle of *Polystoma* limit the production of infective stages of the parasite to the time of year when tadpoles are developing.

V. SUMMARY

1. The formation of *Opalina* cysts in *Rana temporaria* was compared, during September to December, in groups of untreated frogs exposed to normal seasonal fluctuations in temperature, and treated frogs kept at room temperature. Cyst formation was enhanced, to the same degree, in frogs injected with saline or chorionic gonadotrophin (Pregnyl), and, to a higher degree, in frogs injected with frog pituitaries.

2. Observations were made, during September to December, on the state of the testes in *Rana temporaria* infected with *Opalina*. The presence of *Opalina* cysts was most closely correlated with spermiation.

3. Possible factors affecting the formation of opalinid cysts are discussed. It is concluded that the seasonal production of cysts by *Opalina* is not due to cyclical changes inherent in the protozoa. The factor or factors provoking encystation appear to be correlated with the reproductive cycle of the host, and may be the gonadal hormones.

I am most grateful to Dr A. Bishop for much advice and encouragement. I also wish to thank Dr P. Tate for criticizing the manuscript; Professor J. D. Smyth for making available an English translation of the paper by Miretski; and Organon Laboratories Ltd. for a gift of Pregnyl.

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A new species of *Amplicaecum* (Nematoda) from the carpet snake (*Morelia argus variegatus*): with a re-definition and a key for the genus

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Two ascaridoid nematodes have been reported from the carpet snake (*Morelia argus variegatus*), namely *Ophidascaris filaria* and *Polydelphis anoura* (Johnston & Mawson, 1948, 1951). The present paper records a third species, named after Dr F. H. S. Roberts, Veterinary Parasitology Laboratory, C.S.I.R.O., Yeerongpilly, Brisbane.

Amplicaecum robertsi, sp.nov.

Host. Morelia argus variegatus.

Location. In the anterior part of the alimentary tract, usually the stomach. The worms occur in clusters, with their anterior ends deeply embedded in a common opening (Fig. 20). *Ophidascaris filaria* frequently shares the same site of attachment, whereas *Polydelphis anoura* occurs in the intestine.

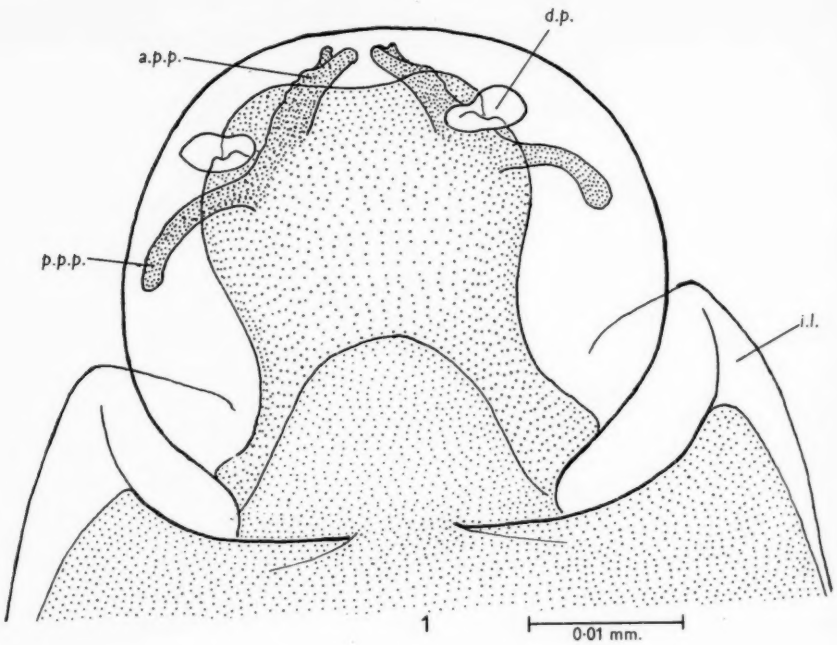
Locality. Coastal Queensland.

Description. The colour, which varies from pink to brown, results from a combination of the deep red body fluid and the brown intestine. Viewed laterally, the body is marked by a herringbone pattern, which serves to distinguish this species from *Ophidascaris filaria*, which has a reticulare pattern. The pattern results from the arrangement of the folds in the intestinal surface.

There are three well-defined lips, whose free border is almost circular in external view (Fig. 1). Between each lip there is an interlabium, whose outline in external view is a triangle with two concave sides (Fig. 2). The bases of the interlabia are external to and considerably wider than the bases of the lips, so that, in surface view, the base of each lip is overlapped by the bases of the interlabia (Fig. 1).

Each lip has three free surfaces, the external surface and two internal surfaces. The external surface of the dorsal lip bears two large external papillae, each containing two prolongations of the pulp representing the dorso-dorsal and latero-dorsal papillae. The fused papilla is pear-shaped in outline, the long axis being transverse and the narrow end being medial (Fig. 1). The external surface of each subventral lip (Fig. 2) bears (1) an external papilla in the ventro-lateral position, resembling the external papillae of the dorsal lip and representing the fused ventro- and latero-ventral papillae, (2) a smaller single papilla in the lateral position, i.e. the ventro-lateral papilla, (3) the amphidial pore, which lies adjacent to and dorsal to the ventro-lateral papilla. The amphidial canal extends posteriorly through the pulp of the lip.

The internal surfaces of the lips are separated from each other by a gutter-like



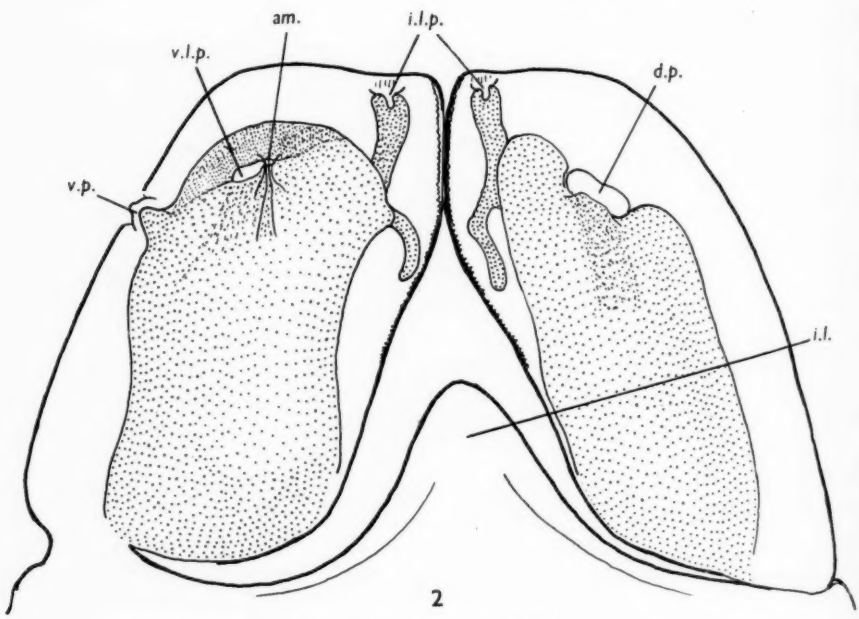


Fig. 1. Dorsal view of dorsal lip.

Fig. 2. Ventral view of subventral lip.

groove (Fig. 3), extending from the margin of the lip to the point where the lip is joined to the body. The sides of the groove become progressively deeper posteriorly, and the free edges converge until they meet. At this point the groove is overlain by the prominent cuticular border of the median lobe of the lip (Fig. 3). In *en face* view the sides of the groove show as two cuticular processes between the internal surfaces (Fig. 4).

The dentigerous ridge extends round the margin of the lip to a point just behind the widest part of the lip (Fig. 2). The denticles are very small, elongate and somewhat irregular in width. On the anterior border of each lip, at the points where the margin turns into the ventral groove, are two minute depressions (Fig. 3). These are probably vestiges of internal labial papillae.

The main pulp of the lips (eulabium) is more or less square in outline; it bears the external papillae and contains, on the subventral lips, the amphidial canal. At each of the anterior corners of the pulp there are two prelabial digitations arising from a short, wide stalk on the internal surface (Fig. 3). The structure somewhat resembles an ice-axe in profile. The anterior digitation passes anteriorly and tends towards the midline; its extremity in lateral view appears bifid and at the tip lies the internal labial papilla (Fig. 2). The posterior digitation is longer; it passes backwards along the border of the lip and terminates at about the widest part of the lip. Between the two digitations there are very fine striations from the pulp to the labial border (Fig. 3).

The mouth communicates directly with the oesophagus. The latter narrows slightly in the region of the nerve ring and gradually increases in width posteriorly, reaching its widest part about 0·5 mm. in front of its junction with the intestine. Posterior to this point it narrows gradually until it is about the same width as the intestine. At the junction, the cuticular lining is continued into the intestine, forming an oval vestibule.

There is no ventricular region at the posterior end of the oesophagus. The dorsal oesophageal gland appears to be limited to the dorsal sector; its nucleus is larger than the nuclei of the subventral glands; it is oval and lies transversely across the dorsal sector (Fig. 5). The nuclei of the subventral glands lie slightly anterior to the dorsal nucleus, in the medial part of each subventral sector (Fig. 5). The ducts of the subventral glands open just anterior to the position of the subventral nuclei, but the glands extend the whole length of the oesophagus. The dorsal gland extends the whole length of the oesophagus and the duct opens into the oesophagus just behind the lips.

The intestinal caecum is considerably narrower than the oesophagus (Fig. 6) and is variable in length (Figs. 7–9); it arises from the dorsal aspect of the intestine, but usually lies laterally to the oesophagus It is slender and gradually tapers to a conical tip which lies at a variable distance from the posterior end of the oesophagus. Some specimens were observed to have two or three caeca (Fig. 10). In some specimens it was barely discernible or absent.

The lining of the intestine is thrown into characteristic transverse folds which give the internal surface an iridescent crimped appearance. On the external surface of the body, these ridges give a herring-bone appearance in lateral view.

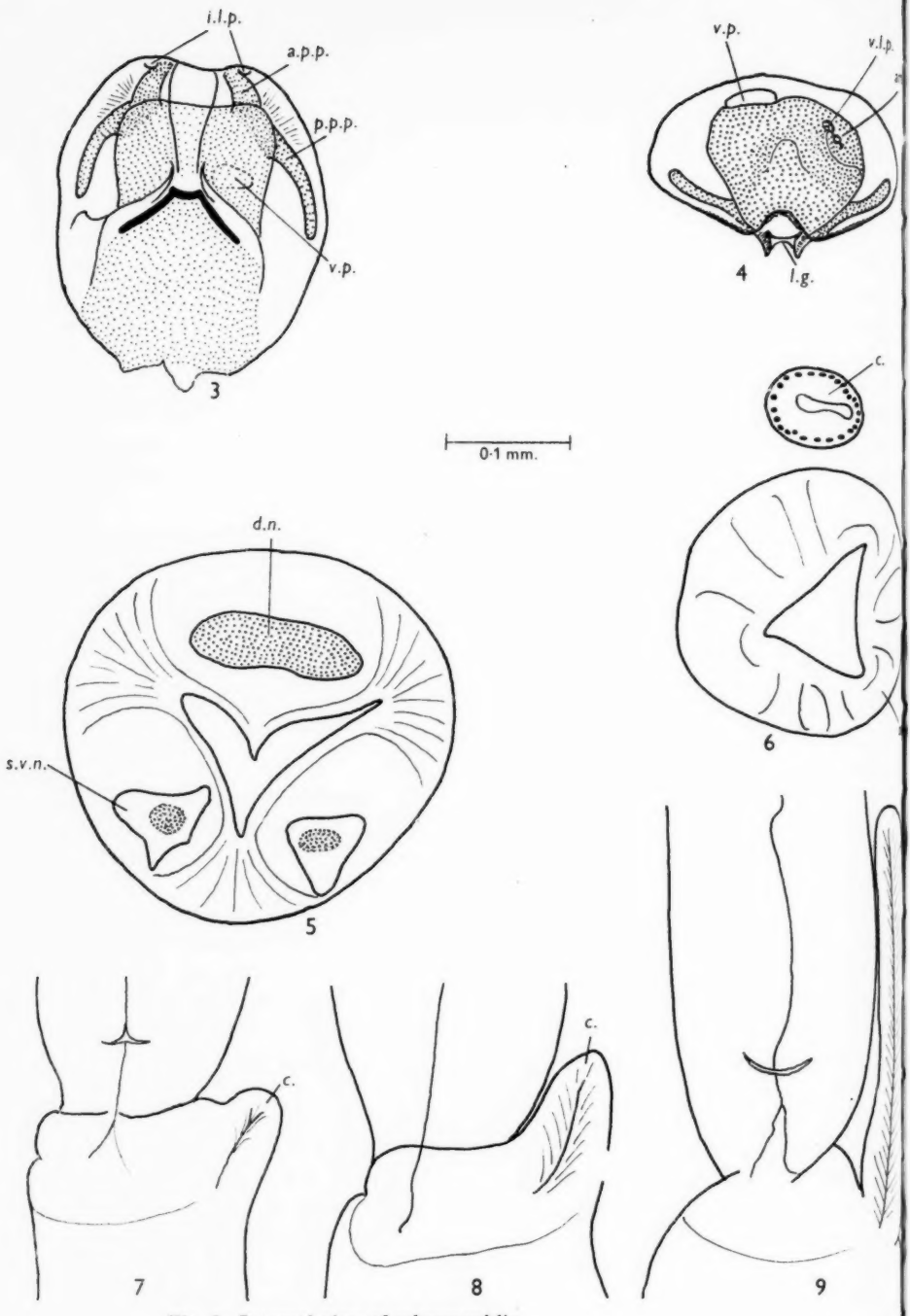


Fig. 3. Internal view of subventral lip.
Fig. 4. *En face* view of subventral lip.
Fig. 5. Nuclei of oesophageal glands.
Fig. 6. Section through caecum and oesophagus.
Figs. 7–9. Intestinal caecum, showing length in different specimens.

The excretory pore is situated in a cup-shaped depression of the cuticle: the excretory duct is usually dilated and has a diameter of 0·04 mm. The excretory nucleus lies in a dilation of the left lateral excretory process. It is oval and measures 0·14 × 0·23 mm.

The deirids (cervical 'papillae') are situated on each side in the mid-lateral line, posterior to the position of the excretory pore. Each appears as a minute cup-like depression of the cuticle, connected with the parenchyme by a fibrous strand (Fig. 11).

Along the mid-lateral line, within the cuticle on each side of the body, extending posteriorly from just behind the lips, are two plates which form a V-shaped structure in cross-section, the point of the V being directed externally. In front of the nerve ring there is no ridge (Fig. 12), but behind it these plates become more robust and push the cuticle into a narrow ala on each side of the body (Fig. 13). Though the alae are only 0·01 mm. at their widest point, which is at the level of the posterior end of the oesophagus, their structure shows that they are true cervical alae.

The body of the male gradually increases in width from the anterior end; it is widest in the middle third and then begins to taper posteriorly. The male reproductive organs occupy the posterior 27–30 % of the body. They are mostly restricted to the left side of the body and consist of a gonadial filament which leads into a coiled tubular vas efferens. The latter terminates in a small bulb and enters the seminal vesicle, which is a capacious thin-walled sac narrowing posteriorly until it becomes a thick-walled tube lined by columnar cells. This tube enters a thick-walled vas deferens which passes backwards towards the rectum (Fig. 14).

In ventral view the tail of the male tapers to a conical point at the tip of which is a short digitiform extension. Just anterior to the cloacal opening is a prominent circular button-like plaque. (It cannot be regarded as a papilla, as no nerve-endings were observed.) There are two subventral post-anal papillae and two lateral post-anal papillae on each side near the tip of the tail. Ventral and posterior to the posterior lateral papilla, lies the phasmid; it differs from the papillae in that it is not raised, but appears as a depression from which a minute canal runs into the parenchyme. In lateral view the hind end of the male is curved ventrally and gradually tapers to a rounded tip (Fig. 17). There is no terminal spike.

The cloaca opens on a mound which is slightly roughened behind the opening, and in front is raised into a ridge with the button-like summit in front of the opening. Just behind and on each side of the cloacal opening there is a double papilla (Fig. 17). There are 52–77 pairs of pre-anal papillae, the first being about 5 mm. behind the anterior end of the spicules when they are withdrawn; the last pair are slightly anterior to the cloacal opening.

The spicules are very long and filiform; they consist of a central rachis and two membranous alae; the anterior end is sharply swollen into a funnel-like knob 0·15 mm. across (Fig. 15). The central shaft of the spicules is about 0·025 mm. wide along most of the length; the alae are about 0·05 mm. wide. The tips of the spicules are slightly roughened, rounded, and not expanded (Fig. 16).

The females are larger than the males; in the anterior half of the body the width

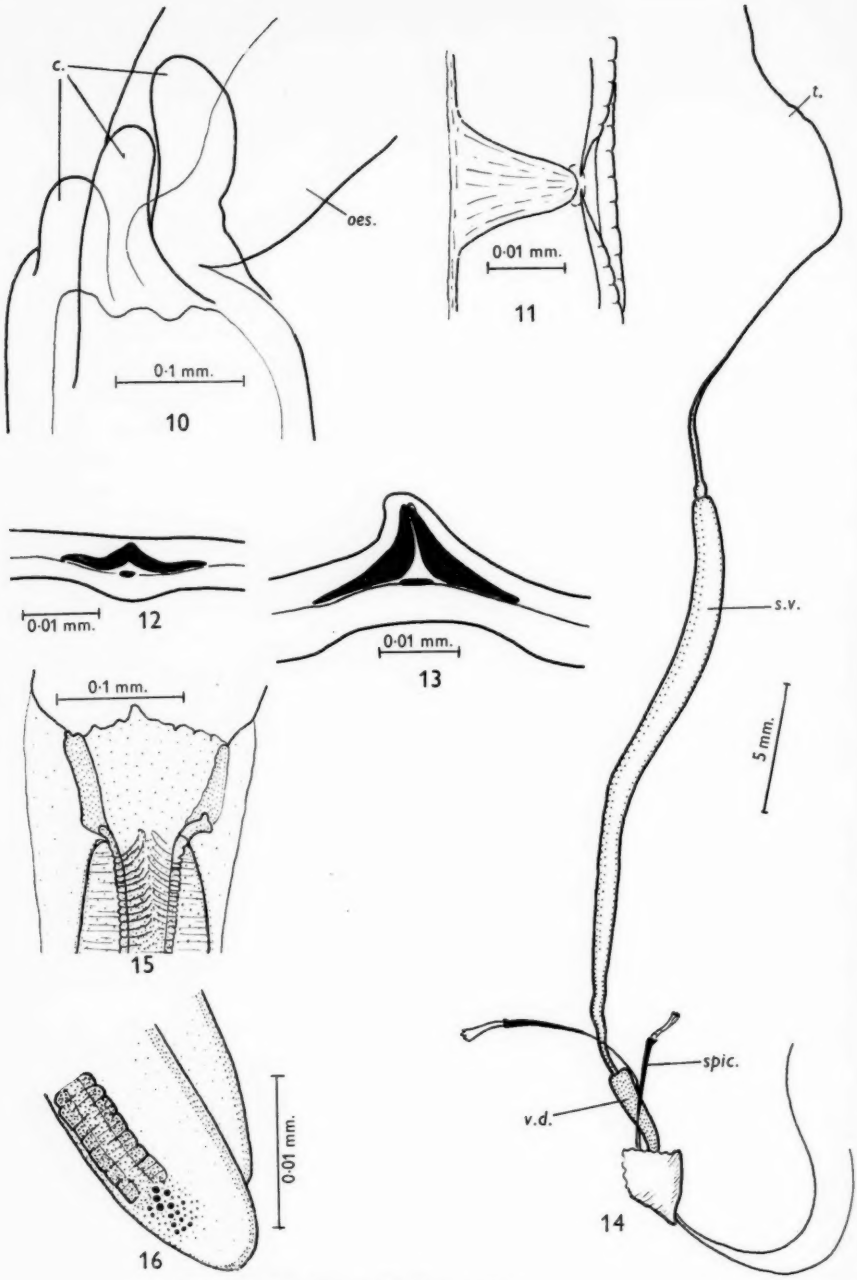
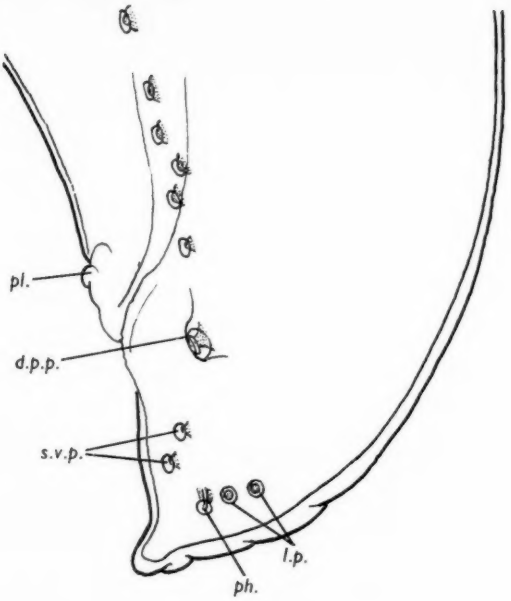


Fig. 10. Specimen with three caeca.
Fig. 11. Deirid.
Fig. 12. Cervical ala at region of nerve ring.
Fig. 13. Cervical ala at region of oesophageal nuclei.
Fig. 14. Male reproductive system.
Fig. 15. Proximal end of spicule.
Fig. 16. Distal end of spicule.



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0·1 mm.

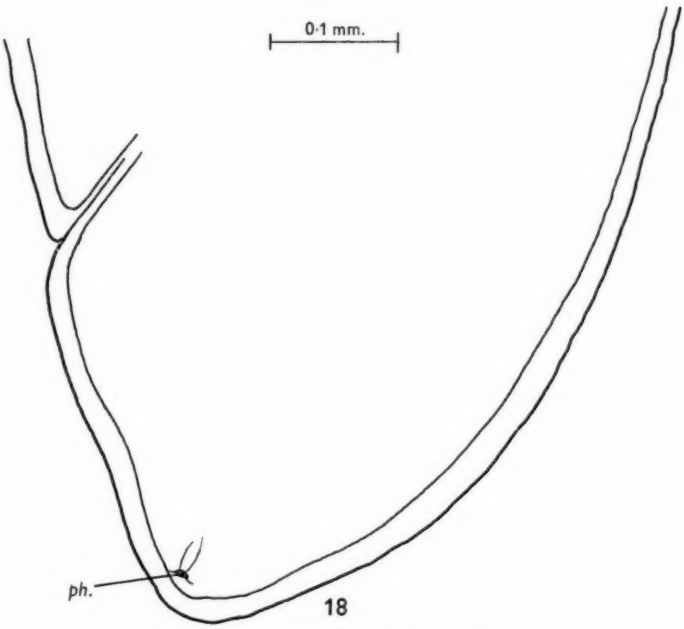

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Fig. 17. Lateral view of tail of male.

Fig. 18. Lateral view of tail of female.

gradually increases and then narrows slightly in the region of the vulva, which lies in the anterior quarter of the posterior half of the body. The posterior half of the body is wider than the anterior half and maintains its width almost to the posterior end.

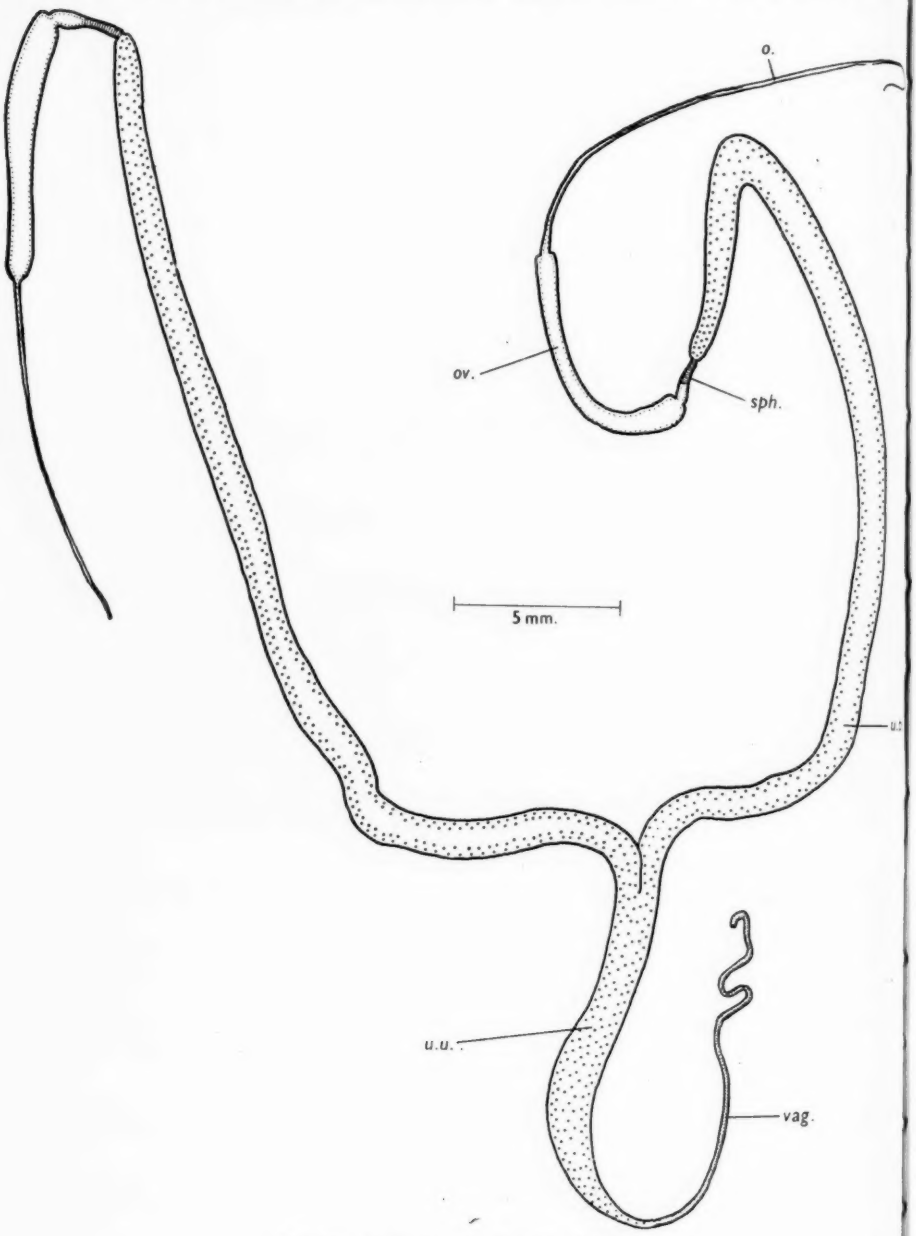

Fig. 19. Reproductive system of female.

The female reproductive organs (Fig. 19) are confined to the posterior half of the body, the vulva being situated slightly in front of or at the level of the most anterior coils of the oviducts. The reproductive organs are largely confined to the left and ventral parts of the body. The vagina is a relatively long, thick-walled, coiled tube, measuring about 13 mm. in length; it meets the undivided portion of the uterus which measures about 12 mm. The latter divides into two branches which extend for about 26 mm. Posteriorly each uterine branch constricts into a narrow thick-walled tube, which again widens abruptly into an elongate thin-walled sac which narrows and continues as the coiled ovary. Two prominent coils extend forward to the region of the vulva and pass back again to the posterior end of the body where the fine coils of the ovaries are situated. The tail of the female is bluntly conical and near the tip lies a pair of phasmids (Fig. 18).

Fig. 20. *Amplicaecum robertsi* in stomach of carpet snake.

The eggs are oval or subspherical and measure 0·079–0·105 × 0·066–0·095 mm.; the surface is pitted with round or oval indentations. When passed in the faeces they contain a spherical cluster of cells.

Life history and development. Work in progress has shown that the eggs of *A. robertsi* hatch when swallowed by rodents, and that the larvae migrate to the liver where they moult for the second time about 5 days after infection. The third-stage larvae may grow to a length of over 100 mm. in the liver of rats. Experimental infection has been produced in laboratory mice and rats, as well as in *Rattus assimilis*, and *R. rattus*, *Melomys cervinipes*, guinea-pigs, and bandicoots (*Thylacis obesula*). Third-stage larvae from infected rodents were fed to carpet snakes and were found to undergo development in the intestinal wall and viscera.

Species differentiation. The genus *Amplicaecum* was proposed by Baylis (1920) to include those ascaridoids which possessed a wide intestinal caecum, small inter-

labia, and lips with dentigerous ridges, but possessed no oesophageal ventriculus, bulb, or appendix. The type species was *A. colurum* (Baylis, 1919) from an African eagle, *Lophoaetus occipitalis* in East Africa.

Eleven species belonging to this genus are now recognized. Except for the type, they are all from amphibia and reptiles, and are all, except *A. alatum*, from the Old World:

A. alatum Baylis, 1947, from *Tupinambis nigropunctatus* in Surinam.

A. cacopi Chatterji, 1936, from *Cacopus systoma* in Burma.

A. excavatum (Hsü & Hoeppli, 1931) Hsü & Hoeppli, 1938, from *Agkistrodon halys brevicaudus* in China.

A. involutum (Gedoelst, 1916) Yorke & Maplestone, 1926, from *Chamaeleon dilepis* in Belgian Congo, also recorded by Baylis (1940) from *Rana nutti*, *Natrix olivacea*, and *Breviceps macrodactylus* in East Africa, and *Cinixys erosa* in Belgian Congo.

(= *A. africanum* Taylor, 1924, from *Bufo regularis* in West Africa.)

(= *A. causi* Thwaite, 1926, from *Causus rhombeatus* in West Africa.)

(= *A. gedoelsti* Yorke & Maplestone, 1926, from toad in Belgian Congo.)

(For synonymy see Baylis, 1940.)

A. mackerrasae Thomas (in the Press) from *Varanus varius* in Queensland.

A. monitor Khera, 1954, from *Varanus monitor* in India.

A. novempapillatum Sandground, 1933, from *Astylosternus robustus* in West Africa.

A. numidicum (Seurat, 1917) Chabaud & Campana-Rouget, 1955.

(= *A. brumpti* Khalil, 1926) from *Rana esculenta* in Corsica and *R. ridibunda* in France.

A schikhobalovi Mozgovoy, 1950, from *Coluber ravergieri* and *Natrix natrix* in U.S.S.R.

A. schoutedeni Baylis, 1940, from *Varanus niloticus* in Belgian Congo.

A. varani Baylis & Daubney, 1922, from *Varanus salvator* in India.

(?) *A. chrysanthemoides* (Skrjabin, 1916) Hartwich, 1957, from *Bufo* spp. in East Africa.

A. robertsi is the only species in the genus to be recorded from a pythonid snake; it is also the longest species yet described and possesses the longest spicules. It differs from all species, except *A. alatum*, *A. excavatum* and *A. schikhobalovi*, in that the vulva is situated posterior to the middle of the body. With regard to these three species, *A. robertsi* differs clearly from *A. alatum*, first, in that the cervical alae are only one-tenth as wide as in the latter species, and secondly in that the intestinal caecum is less than a quarter of the length of the oesophagus, whereas in *A. alatum* it is more than half the length of the oesophagus. It differs from *A. excavatum* and *A. schikhobalovi* in regard to the shape of the lips, body length, position of the vulva, and the spicule length. The lips of *A. robertsi* are almost circular, whereas those of the other two species are trapezoidal. The body length of *A. schikhobalovi*, according to Mozgovoy (1953), is 35–38 mm. for the male and 55–62·5 mm. for the female; the spicule length is 2·03–2·13 mm. The body length of *A. excavatum*, according to Hsü & Hoeppli (1931), is 76–93 mm. for the male and

60–126 mm. for the female; the spicule length is 2·00–4·6 mm. Corresponding measurements for *A. robertsi* are shown in Tables 1 and 2. The position of the vulva in *A. excavatum* and *A. schikhobalovi* is in the posterior quarter of the body, whereas in *A. robertsi* it is situated in the first quarter of the posterior half of the body.

Table 1. *Measurements in mm. of the type specimens of* Amplicaecum robertsi *sp.nov.*

	Holotype (male)	Allotype (female)
Length	132	157
Width (at posterior end of oesophagus)	0·788	0·882
Dorsal lip (length)	0·176	0·171
Dorsal lip (width)	0·186	0·201
Interlabia (length)	0·083	0·083
Subventral lips (length)	0·201	0·201
Nerve ring (from anterior end)	0·788	0·706
Excretory pore (from anterior end)	1·176	1·118
Cervical papillae (from anterior end)	1·293	1·070
Oesophagus (length)	5·43	5·57
Oesophagus (width at widest point)	0·388	0·517
Caecum (length)	0·506	0·706
Vulva (distance from anterior end)	—	93
Anus (to tip of tail)	—	0·306
Phasmids (to tip of tail)	—	0·039
Spicules	13·1	—
Cloaca (to tip of tail)	0·235	—
Number of pre-anal papillae	68 pairs	0
Number of post-anal papillae	4 pairs + 1 double pair	0
Number of phasmids	1 pair	1 pair

* Type specimens deposited at Queensland Museum, Brisbane; paratypes at the Department of Parasitology, University of Queensland, Brisbane.

A key to the differential features of the genus *Amplicaecum* is given below:

Vulva anterior to middle of body.
  Caudal alae conspicuous.
    10–15 pairs of preanal papillae.
      Teeth in dentigerous ridges large and coarse; spicules 0·7—a little over 1·0 mm.; cloaca not on conspicuous elevation. *A. involutum* (= *A. novempapillatum*)

      Teeth in dentigerous ridges extremely small; spicules 0·55–0·66 mm.; cloaca on conspicuous elevation. *A. schoutedeni*

    Five pairs of pre-anal papillae.
      Male 11–19 mm., female 14–35·5 mm., 4 pairs of postanal papillae, vulva at junction of anterior and middle thirds. *A. cacopi*

      Male 44 mm., female 70 mm., three pairs of postanal papillae, vulva at junction of first and second quarters. *A. numidicum*

  Caudal alae inconspicuous or absent.
    Caecum about half length of oesophagus, spicules more than 1 mm. *A. mackerrasae*

Caecum about quarter length of oesophagus, spicules 0·5 mm. or less
Median precloacal papilla present. *A. varani*
Median precloacal papilla absent. *A. monitor*

Vulva posterior to middle of body.
Cervical alae conspicuous (width 0·1–0·12 mm.); intestinal caecum more than half length of oesophagus. *A. alatum*
Cervical alae inconspicuous or absent, intestinal caecum less than half length of oesophagus.
Lips circular in outline, vulva just behind mid-point of body, spicules 6·6–15·8 mm. *A. robertsi*
Lips trapezoidal in outline, vulva in posterior quarter of body, spicules less than 6 mm.
Body length: male 76–93 mm.
female 60–126 mm. *A. excavatum*
Body length: male 35–38·5 mm.
female 55–62·5 mm. *A. schikhobalovi*

Table 2. *Range of measurements in mm. of paratypes of* Amplicaecum robertsi

	Males	Females
Number of specimens	6	8
Length	70–152	117–181
Width (at posterior end of oesophagus)	0·494–0·823	0·740–1·11
Dorsal lip (length)	0·122–0·196	0·157–0·230
Dorsal lip (width)	0·122–0·220	0·176–0·245
Interlabia (length)	0·073–0·088	0·069–0·106
Subventral lips (length)	0·127–0·191	0·157–0·265
Nerve ring (from anterior end)	0·482–0·823	0·611–0·847
Excretory pore (from anterior end)	0·576–1·160	0·823–1·270
Cervical papillae (from anterior end)	0·694–1·446	1·037–1·670
Oesophagus (length)	3·71–6·24	4·35–6·14
Oesophagus (width at widest point)	0·353–0·505	0·470–0·600
Caecum (length)	0·294–0·823	0·343–0·823
Vulva (distance from anterior end)	—	72–95 (52–62% body length)
Anus (to tip of tail)	—	0·306–0·470
Phasmids (to tip of tail)	—	0·034–0·059
Spicules	6·58–15·8	—
Cloaca (to tip of tail)	0·122–0·206	—
Number of pre-anal papillae	52–77 pairs	0
Number of post-anal papillae	4 pairs + 1 double pair pair	0
Number of phasmids	1 pair	1 pair

The type species, *A. colurum*, is not included in the key because only the female is known. According to Baylis (1947) it was probably a pseudo-parasite, the real host being an amphibian or reptile. The synonymy proposed by Baylis (1940) for *A. involutum* is adopted in the key, although Mozgovoy (1953, p. 485) questioned this synonymy on the grounds that the hosts of *A. involutum* sensu Baylis, 1940, included both amphibia and reptiles, Nevertheless, it is evident from Baylis's observations on the actual specimens, that the species involved cannot be differentiated morphologically. Hence for taxonomic purposes *A. gedoelsti*, *A. causi* and *A. africanum* should be regarded as synonyms of *A. involutum*.

It will be observed that *A. novempapillatum* Sandground, 1933, is regarded as a synonym of *A. involutum*. According to the descriptions of Baylis (1940) and Sandground (1933) the only differences between these two species are as follows:

	A. involutum	*A. novempapillatum*
Spicules (length)	'A little over 1·0 mm.'	1·15 mm.
Preanal papillae	10–13	9
Postanal papillae	4	3 + 'caudal gland'

By 'caudal gland' Sandground presumably meant phasmids: these structures closely resemble papillae, and Baylis does not differentiate between them. The sole difference evidently resides in one preanal papilla, a difference which is not regarded by the present writers as denoting specific distinction. Without access to the type specimens such synonymy can only be regarded as subjective.

A. monitor Khera, 1954, is differentiated from *A. varanus* Baylis & Daubney, 1922, because Khera was unable to find a median preanal papilla in the male. He was also unable to find the two 'papillae' on the tail of the female mentioned by Baylis & Daubney; these are no doubt the phasmids and, as they are almost universally present on the female tail in ascaridoids, this distinction is regarded as of doubtful significance. The other differentiating features mentioned by Khera are not regarded as specific distinctions by the present writers.

A. schikhobalovi Mozgovoy, 1950, appears to resemble very closely *A. excavatum* (Hsü & Hoeppli, 1931). The former has three pairs of lateral postanal papillae (one pair are probably phasmids), the latter has two pairs of lateral postanal papillae (probably the phasmids were present, but not observed). Apart from the body length, no differential features of specific value could be detected from the descriptions. Mozgovoy (1953) did not include *A. excavatum* in his key to the genus.

DISCUSSION

The genus *Amplicaecum* was included by Baylis (1920*a*) in the subfamily Anisakinae Railliet & Henry, 1912, emend. Baylis, 1920. Skrjabin & Karokhin (1945) proposed the family Anisakidae to include the subfamily Anisakinae and a subfamily Angusticaecinae, in which they included *Amplicaecum* and the closely allied genus *Augusticaecum* Baylis, 1920. Mozgovoy (1953) raised the Angusticaecinae to the status of a family, i.e. Angusticaecidae, and included a third genus, namely *Metangusticaecum* Mozgovoy, 1953. He separated the Angusticaecidae from the Anisakidae and included both families in the superfamily Anisakoidea.

The classification proposed by the Russian workers, though it has served to emphasize the differential characters of the ascaridoids in general, has tended to create wide separation of what are evidently closely related species. Thus, *Ophidascaris mombasica* was regarded by Hsü & Hoeppli (1931) as very similar to *O. excavatum*, which they later (Hsü & Hoeppli, 1938) discovered possessed a small intestinal caecum and hence removed it to the genus *Amplicaecum*. Similarly, *A. robertsi* was originally identified by the writers as *Ophidascaris filaria*. (The caecum was first discovered in the larvae, because it is relatively more conspicuous.)

Nevertheless, according to Mozgovoy's classification, *Ophidascaris filaria* and *Amplicaecum robertsi* belong to different superfamilies, i.e. the Ascaridoidea and Anisakoidea respectively. It is evident that the morphological differences between these two species are relatively slight (unpublished observations).

Hartwich (1957) has proposed a system of classification of the ascaridoids, in which the ascaridoids of amphibia and reptiles are included in two subfamilies of the family Ascarididae, namely the Ophidascaridinae Hartwich, 1954, and the Angusticaecinae Skrjabin & Karokhin, 1945. Recently Osche (1958) has proposed the family Angusticaecidae to include these two subfamilies and to separate them from the Ascarididae. Thus Angusticaecidae sensu Osche differs from Angusticaecidae sensu Mozgovoy, in that the latter does not include the Ophidascaridinae.

Hartwich (1957, p. 220) has defined the features of the genus *Amplicaecum* as follows: '*Angusticaecinae*—Lippen vier- oder sechseckig, ohne oder mit schwach entwickelten Labialflügeln. Kleine Interlabien vorhanden. Halsflügel fehlend (nur bei *A. alatum* Baylis, 1947, vorhanden). Dorsales Darmcaecum (bei *A. africanum* Taylor, 1924, paarig entwickelt) fast ebenso dick wie Ösophagus. Spicula gleich oder fast gleich lang. Vulva vor der Körpermitte liegend (nur bei *A. alatum* dahinter). Parasiten von Amphibien und Reptilien.'

To include *A. robertsi*, this definition would require modification, to comply with the following:

(1) The lips of *A. robertsi* were circular in outline.

(2) On superficial examination *A. robertsi* would be described as being devoid of cervical alae, but on section it is apparent that these structures are present, although very narrow. It appears that the only other description of a species of *Amplicaecum*, which is accompanied by an illustration of a section of the cervical region, is that of *A. alatum* Baylis, 1947, which clearly possesses cervical alae, and which Hartwich cites as the only species possessing them. It seems probable that, on section, other species may be found to possess cervical alae.

(3) In *A. robertsi* the caecum was usually considerably narrower than the oesophagus; according to the figures this was also the case with *A. alatum* Baylis, 1947, *A. monitor* Khera, 1954, and *A. schikhobalovi* Mozgovoy, 1950.

(4) In *A. robertsi* the vulva was behind the middle of the body, as was also evidently the case with *A. alatum*, *A. excavatum* and *A. schikhobalovi*.

Furthermore, there are other described species which do not fit Hartwich's definition, e.g. *A. numidicum* appears from Khalil's figures to have well-developed 'labial wings' and the same might be said of *A. schoutedeni* from Baylis's figure.

Nevertheless, as no other species within the genus *Amplicaecum* are available for study, the following re-definition can only be regarded as tentative:

Lips variable in shape, dentigerous ridges present. Interlabia present, cervical alae present or absent. Intestinal caecum usually present. Spicules equal or subequal. Parasites of amphibia and reptiles (except for the type species *A. colurum*).

It is evident that when these modifications are made, the only feature which differentiates the genus *Amplicaecum* from the genus *Angusticaecum* is the presence of interlabia in *Amplicaecum*.

It would appear that the groups proposed by Hartwich (1954, 1957) and Osche (1958) are more acceptable in the phylogenetic sense than those adopted by Mozgovoy (1953). Nevertheless, it is clearly necessary to establish them on a more secure morphological basis by (1) determination of the phylogenetic significance of each feature upon which differentiation is already based, and (2) more detailed comparative anatomical studies, to determine whether there are other, possibly more significant, differences to be taken into account. Thus, Osche's proposal (Osche, 1958) that the ascaridoids of amphibia and reptiles should be grouped in a separate family, i.e. Augusticaecidae, from those of mammals, would appear to be sound, particularly in view of their different hosts and their different method of feeding. But its adoption depends upon more detailed morphological studies, so that the two families can be more distinctly separated on a morphological basis.

With regard to the subdivision of the family Angusticaecidae into subfamilies, there would appear to be no more justification for grouping on the basis of the intestinal caecum, than on characters such as the presence of interlabia or the number of uterine branches. On examination of Hartwich's definitions (Hartwich, 1957), the only distinctive feature differentiating the two subfamilies Ophidascaridinae and Angusticaecinae is the presence in the latter of an intestinal caecum. But this is not a valid distinction, as this structure is barely discernible in some species of *Amplicaecum*, e.g. *A. robertsi*, and is present in some species included in the Ophidascaridinae, e.g. *Hexametra waterstoni* (Baylis, 1920).

SUMMARY

A new species of *Amplicaecum* is described from the carpet snake (*Morelia argus variegatus*) in coastal Queensland. It differs from all previously described species in this genus in that its host is a pythonid snake. It is the largest species yet described, and the spicules are about 3 times as long as in any other species. The genus *Amplicaecum* is tentatively re-defined and a key is given to the differential features of the recognized species. The view is expressed that before classification of these and related ascaridoids can be adopted, further information is necessary, particularly on (1) the phylogenetic significance of differential features already in use, and (2) on the existence of other more significant differential features.

LIST OF ABBREVIATIONS

am.	amphid
a.p.p.	anterior prolongation of pre-labial pulp
c.	intestinal caeca
d.p.	fused dorso-dorsal and latero-dorsal papillae
d.n.	nucleus of dorsal oesophageal gland
d.p.p.	double post-cloacal papilla
i.l.	interlabium
i.l.p.	internal labial papilla
l.p.	lateral post-cloacal papilla
l.g.	labial groove
o.	ovary
ov.	sac-like region of ovary
oes.	oesophagus
p.p.p.	posterior prolongation of pre-labial pulp
ph.	phasmid
pl.	pre-cloacal plaque
s.v.	seminal vesicle
s.v.n.	nucleus of subventral oesophageal gland
s.v.p.	subventral post-cloacal papilla
spic.	spicule
sph.	sphincter between ovary and uterus

LIST OF ABBREVIATIONS (*cont.*)

t.	testis	*v.p.*	fused latero-ventral and ventro-ventral papilla
u.u.	undivided part of uterus	*v.d.*	vas deferens
u.b.	uterine branch	*vag.*	vagina
v.l.p.	ventro-lateral papilla		

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A review of some ticks (Acarina: Ixodidae) of sea birds

Part II. The taxonomic problems associated with the *Ixodes auritulus-percavatus* group of species

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Cooley & Kohls (1945), in the introduction to their monographic study of the genus *Ixodes* in North America, stated that 'The fact that a close relationship of species is so frequent in *Ixodes*, sometimes involving not two but several species, has resulted in a tendency to describe new forms as subspecies of species already established. In many instances these new forms have been found to be as closely related to some other species as to the one with which they are associated.' The truth of this assertion has been demonstrated in the *Ixodes rasus* group (Arthur, 1957*a*), in the *Ixodes nairobiensis* group (Arthur, 1959) and in the complex of species associated with *I. auritulus* Neumann, 1904, and *I. percavatus* Neumann, 1906, which form the subject of this paper.

Dependable diagnostic morphological distinctions separating species of the genus *Ixodes* are matters of degree rather than the presence or absence of characteristic features. For this reason text or figures are frequently inadequate to make convincing distinctions between species. These distinctions are readily evident when a sufficiently large sample is available, and the possible intraspecific variation ascertained. Difficulties do arise, however, when dealing with a few specimens or with species having a wide geographical range, because we do not know how individuals of a species differ *inter se*. A pertinent example of this is seen in *Ixodes angustus* Neumann, 1899, whose distribution is reported from North America (Cooley & Kohls, 1945), Sakhalin Island (Asanuma, 1951), U.S.S.R. (Pomerantzev, 1950) and possibly from Korea (Arthur, 1957*b*). The nymphs of this species, as described by Cooley & Kohls, have small pointed cornua, but Nuttall & Warburton (1911) show these structures in specimens from British Columbia as long and pointed, as also does Arthur in Korean specimens.

This wide distribution of a species is also true of *Ixodes auritulus*, which has been reported from 'Equateur' (?), Mis Charcot, 'Baie Orange Mission du Cap Horn', Punta Arenas, Straits of Magellan, South America (Neumann, Paris Museum collection); from Alaska, Colorado, Oregon, Canada, Guatemala, Brazil, Peru (Cooley & Kohls); from British Columbia, the Australian Antarctic, Costa Rica, Argentina, Peru (Nuttall & Warburton, 1911; Nuttall, 1916); from Canada, where according to Gregson (1956): 'This distinctive species has a remarkably wide distribution, and occurs on passerine and gallinaceous birds from the most southerly tip of South America north at least to the Queen Charlotte Island, B.C. In Canada it appears to be confined mainly to the Pacific coast, where it replaces

Haemaphysalis chordeilis (Packard) as the common parasite on grouse.' According to Zumpt (1952) *I. auritulus* is a fairly common parasite of sea birds and is 'widely distributed in the southern hemisphere. Its area of distribution nearly equals that of *C. uriae* but though it reaches the northern parts of North America, it has not yet been found in northern Europe and Asia.' Its southerly distribution appears to be confirmed by Dumbleton (1953) who reports it from Snares Island, New Zealand. This distribution may require modification because of inaccurate diagnosis.

These difficulties of diagnosis are aggravated because of the mutilated state of type specimens as in *I. auritulus* and *I. percavatus*, or because of inability to obtain sufficient specimens of a species from one host, or a number of ticks from several hosts of the same species in the same geographical region. Almost invariably the few specimens of ticks received are preserved, so that breeding the species through from egg to adult cannot be done by the investigator. Sometimes, too, specimens may be mounted dry on card which necessitates their removal for complete study, and during this process parts of the legs, palps or even the entire capitulum may be irreparably damaged. Frequently the capitulum may be damaged or completely removed during extrication from the host, and this all too often means that the specimen cannot be identified with any degree of accuracy. Distortion of essential features results from mounting entire ticks on slides and interpretations based on such preparations are extremely misleading. In the present studies the ticks were mounted on a plasticene base and orientated as required under a binocular microscope before being drawn with the aid of a camera lucida.

The separation of Ixodes auritulus *and* Ixodes percavatus *groups. Ixodes* with a large conspicuous palpal article I and a distinct anterior projection have, hitherto, been identified as *I. auritulus*. This view is not, of course, inconsistent with previously published literature, and consequently *I. percavatus* has been synonymized with *I. auritulus* by Zumpt (1952), Arthur (1953) and accepted by Santos Dias (1958). Zumpt based his synonymy on several nymphs and females collected from Nightingale Island without reference to the type (Zumpt, 1952, p. 16), and Arthur on the much mutilated type specimens, lacking palpal segments II and III, hypostome and legs. Nuttall & Warburton (1911) describe the first joint of the palp of *I. percavatus* as 'triangular ventrally, with an anterior point internally and a posterior point', and Zumpt (*loc. cit.*) elaborates further when he points out that the anterior point in the type specimens 'is perhaps not as big or clearly visible as in the unengorged specimens of *I. auritulus*, so that this feature would not be against the synonymising of these two species'.

Having re-examined the types of *auritulus* and *percavatus*, as well as other material, including that of Zumpt, from Nightingale Island, Tristan da Cunha, it must now be accepted that the 'anterior point' on palpal article I of *percavatus* and the 'anterior projection' on the same article in *I. auritulus* are essentially of the same pattern and probably homologous. *I. percavatus* does, however, differ from *I. auritulus* in having the meso-dorsal margin of article I prolonged dorsally to form a strong spur, which is evident both dorsally and laterally. Accordingly, *I. percavatus* should be resurrected as a valid species.

In order to facilitate the identification of these ticks for future workers it is proposed to review earlier descriptions and figures of the types and to supplement them with material collected from their main regions of occurrence. This survey revealed that individual specimens, previously assigned either to *I. auritulus* or to *I. percavatus*, differed quite markedly from the types. For purposes of description I have therefore dealt with these specimens either in the *I. auritulus* group or in the *I. percavatus* group.

IXODES AURITULUS GROUP

I. auritulus type and electotype material (Figs. 1–10*a*). Neumann's type specimens consist of four partly gorged females collected from an unnamed bird at Punta-Arenas, Straits of Magellan, South America by Lebrun. Three of the four type specimens are deposited in the Paris Museum of Natural History (Accession nos. 2474–83), and the fourth in the École Nationale de Vétérinaire, Toulouse. All these specimens are badly mutilated. Accordingly, it is proposed to make an electotype of the female collected at Mis Charcot (1905, no other data) and identified as *I. auritulus* by Neumann in 1907. This electotype is also deposited in the Paris Museum of Natural History.

Female

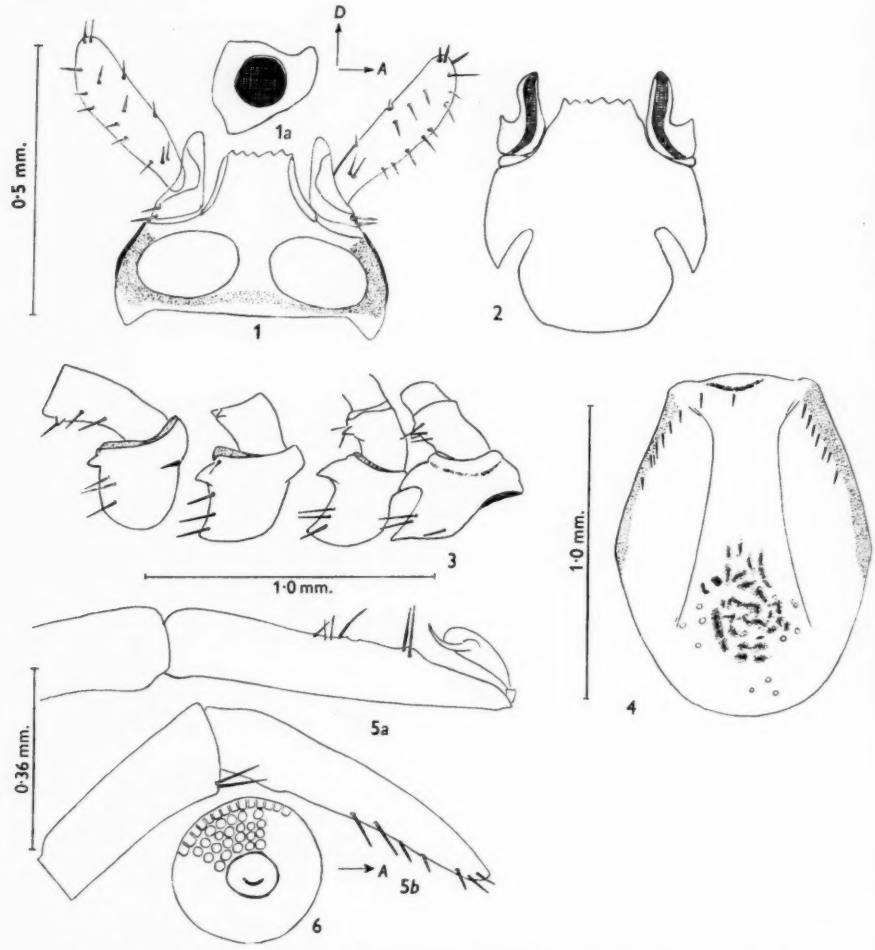
Body. Alloscutum brown; capitulum, scutum and legs straw. Length of partly engorged specimens 6·7–7 mm.; width 5·3–5·5 mm.

Capitulum. Length 0·69 mm.; width 0·52–0·53 mm. Basis about twice as broad as long, surface smooth; posterior margin slightly concave or straight, lateral margins arcuate; cornua well defined, broad, rounded apically and not elevated above the posterior margin. Porose areas large, elongate-oval and separated from each other by an interval of about half the greatest width of one of them. Palpal article I large, triangular dorsally with a weak salience on postero-internal margin (Figs. 7, 7*a*), mesially drawn out to form a horn-like protrusion which has a slight upward curvature distally (Figs. 1*a*, 7*a*). Inner margins of palpal articles II and III almost straight, outer margins gently curved giving a club-like appearance when seen dorsally; combined length 0·37 mm. Sutural line between the articles faint. Basis in ventral view mildly convex and smooth, constricted at the middle, posterior margin almost straight (Figs. 2, 8) and lateral margins curved. A few short spinose hairs postero-laterally and a pair of fine hairs behind the base of the hypostome (Fig. 8). Auriculae as large, broad, tapering horns.

Hypostome. Missing from all specimens. Neumann (1899) and Nuttall & Warburton (1911) stated that the hypostome is 'rounded or truncated distally, of uniform width, bearing 6/6 then 5/5 rows of pointed teeth, the external rows longest'. Neumann's fig. 23 (1899) also shows 1 row of 4/4, 1 row of 3/3 and 1 row of 2/2 files basally.

Scutum. Length 1·17–1·23 mm., width 0·92–0·98 mm. Elongate-oval, broadly rounded behind, antero-lateral margins flattened, widest at about mid-length.

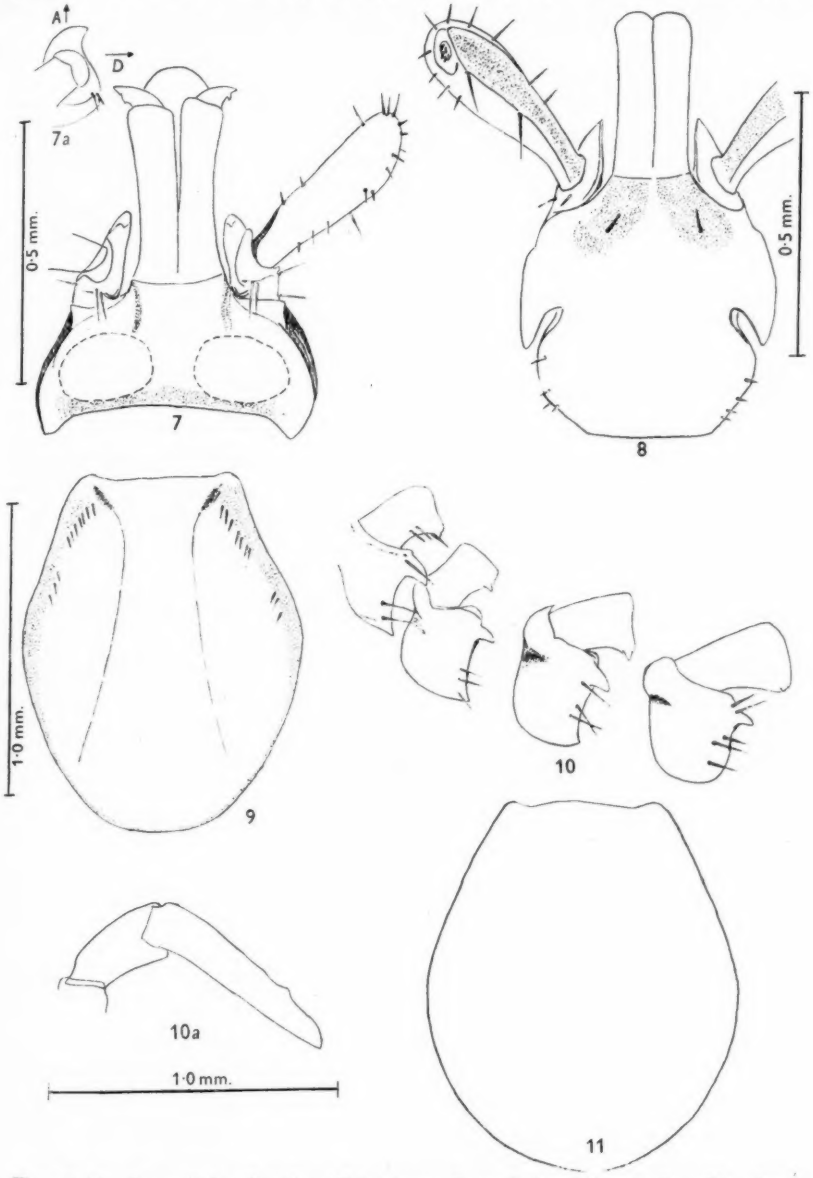
Scapulae much reduced, emargination very slight. Surface wrinkled in postero-median field, smooth anteriorly and beyond the cervical grooves. Cervical grooves arise from a broad shallow depression anteriorly, forming linear depressions more posteriorly, first converging, then diverging and ceasing in front of the postero-lateral edges. Punctations shallow, moderate in number. Hairs few, fine, of moderate length being most numerous anteriorly and antero-laterally. Lateral carinae absent.



Figs. 1–6. *I. auritulus* female, Neumann's 'types': 1, capitulum, dorsal; 1*a*, side view of palpal article 1; 2, ventral view of basis; 3, coxae and trochanters I–IV; 4, scutum; 5*a*, tarsus I; 5*b*, tarsus and metatarsus IV; 6, spiracle.

Legs. Moderate in size, smooth, shining with few hairs. Tarsi long, narrow (Figs. 5*a*, *b* and 10*a*) with mild subapical humps. Length of tarsus I, 0·73 mm., metatarsus I, 0·39 mm. Length of tarsus IV, 0·66 mm., metatarsus IV, 0·45 mm.

Coxae. (Figs. 3, 10). Smooth, convex except for depressions indicated by stippling in Fig. 10. Hairs few and spinose. External spurs on all coxae large, those on II–IV in the electotype of approximately equal size. Internal spur on



Figs. 7–11. *I. auritulus* female. Electotype: 7, capitulum, dorsal; 7*a*, side view of article I; 8, capitulum, ventral; 9, scutum; 10, coxae and trochanters I–IV; 10*a*, tarsus I; 11, Outline of scutum of female, *I. auritulus*, from Peru (Rocky Mountain Laboratory no. 31545).

coxa I of moderate length and sharply tapered, and in Neumann's types directed towards the mid-line: internal spurs on coxae II and III as distinct saliences except in the 'Toulouse specimen' where they are distinct spurs; no internal salience or spur on coxa IV. Trochantal spurs distinct, but definitely shorter than external coxal spurs.

Spiracular plate. Almost circular, surface slightly concave, little elevated above the rest of the integument; macula either central (Neumann's types) or antero-ventral (electotype). Goblets of moderate size, numerous.

Genital aperture. On a level with the intercoxal spaces between legs III and IV.

Anal groove. Rounded in front of anus, parallel sided behind.

COMMENTS ON OTHER SPECIMENS EXAMINED

(*a*) *Other South American specimens readily identifiable with type and electotype*

Two females, from *Trupialis militaris*, San Sebastian Settlement, Tierra del Fuego (Crawshay Collection, October 1906). British Museum of Natural History.

One female, from *Poecilothraupis igniventris*. 3 miles north of Limbani, 3500 ft. Dpto Puno, Peru. C. B. Koford *leg.* 25 October 1951. The form of the scutum differs from the type and is shown in Fig. 11. (Rocky Mountain Laboratory no. 31545.)

One female, from pipit, Lunin, Peru. H. T. Townsend *leg.*, G. H. F. Nuttall collection 2520, deposited in the British Museum of Natural History.

(*b*) *Material previously identified as* I. auritulus *and now assigned elsewhere*

The material consisting of (i) three females 'Parasites trouvé sur une espèce de Caille, Equateur', Dr Rivet *leg.* (Paris Museum), and (ii) a single well-gorged female collected by the 'Baie Orange Mission du Cap Horn', 1882–8, Dr Hyades and Sauvinet, no. 3063–1885 (Paris Museum) was identified by Neumann as *I. auritulus*, but because of the meso-dorsal spur on palpal article I, along with other characters, now comes under the *I. percavatus* group as *I. cornuae* sp.nov. (see later).

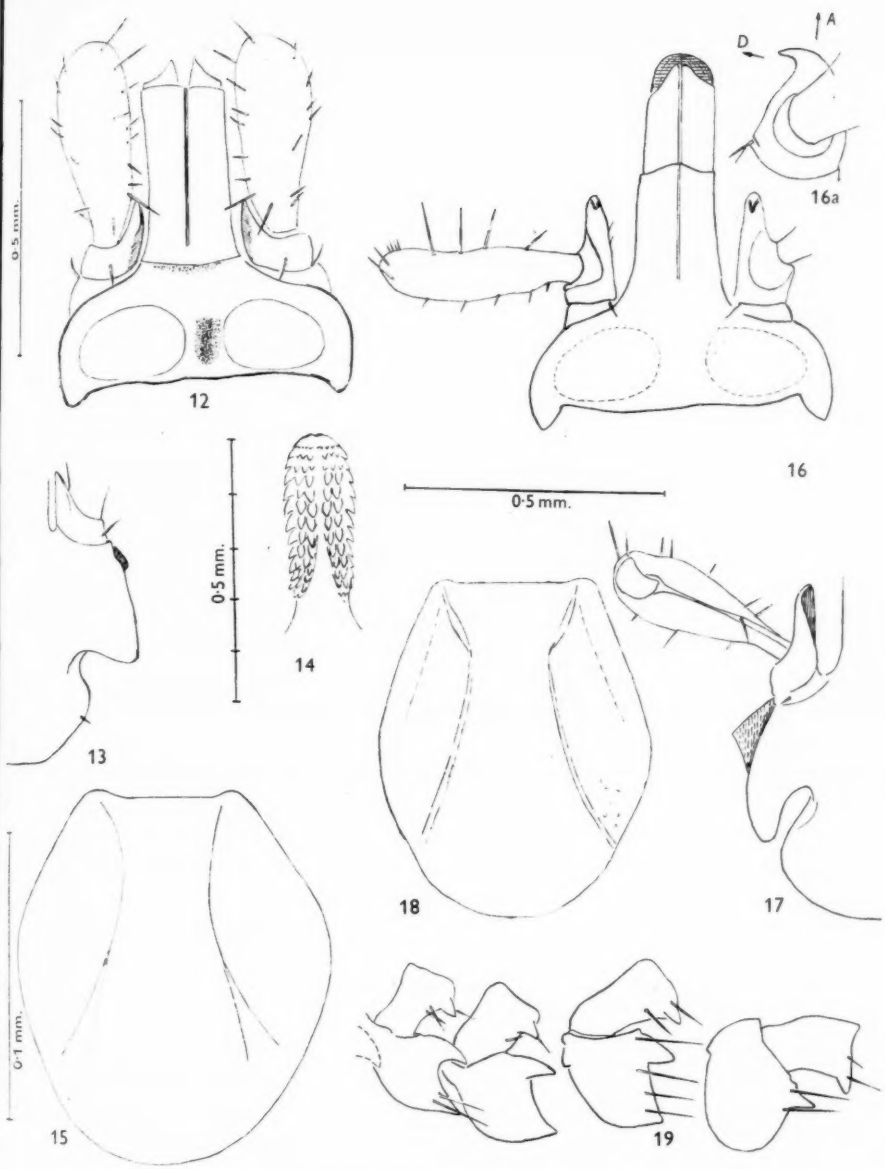
(*c*) *Peruvian specimens*

One female collected from *Caloptes rupicola* S.S.W. Limbani, Dpto Puno, Peru. 1 August 1951 ex Rocky Mountain Laboratory, U.S.P.H.S. Montana no. 31543.

Description of female (Figs. 12–15)

Body. Colour brown, body elongate-oval.

Capitulum. Length 0·69 mm.; width 0·57 mm. Basis 2–3 times as broad as long; posterior margin slightly undulate, salient; cornua broad and elevated (Fig. 12). Lateral margins arcuate. Surface undulate behind the porose areas and depressed between them. Porose areas shallow and transversely ovate. Palpal article I much as in the type and electotype. Palpal articles II and III club-shaped, sutural line between them indistinct, combined lengths 0·37 mm. Hairs strong of moderate length. Basis in ventral view (Fig. 13) smooth, strongly constricted at about mid-length. Posterior margin almost straight and relatively short, postero-lateral margins sharply angled and attaining greatest width just



Figs. 12–19. Figs. 12–15, *I. auritulus* female from Peru (Rocky Mountain Laboratory no. 31543). 12, capitulum, dorsal; 13, basis, ventral; 14, hypostome, note dentition; 15, scutum. Figs. 16–19, *I. auritulus* female from Guatemala (Rocky Mountain Laboratory no. 32153). 16, capitulum, dorsal; 16*a*, side view of palpal article 1; 17, basis, ventral; 18, scutum; 19, coxae and trochanters I–IV.

behind constriction. Auriculae broad, and forming almost a right angle postero-laterally (not horn-like as in types and electotype).

Hypostome. Large, broadly rounded apically, except for slight median indentation. Dentition posterior to small corona as 5/5 apically, one row of 4/4, then 3/3 for most of the length and 2/2 proximally. Denticles broad and sharp, about equal in size in both median and lateral files. Length 0·37 mm.

Scutum. Length 1·28 mm., width 1·12 mm. Suboval with greatest width at about mid-length, broadly rounded behind, flattened along antero-lateral margins. Humeral angles much reduced. Surface smooth, shining; punctations fine, few in number and well dispersed. Cervical grooves as broad depressions anteriorly, somewhat narrower posteriorly, and not reaching postero-lateral margins. Lateral carinae absent.

Legs. Longer and broader than in type specimens.

Coxae. Smooth with few long hairs. Internal spur on coxa I scarcely more than a salience as are those on coxae II and III, absent on IV. External spur on coxa I, heavy, broad and tapered, on coxae II and III short and broad, rounded apically. Trochantal spurs about as long as external coxal spurs on legs II–IV. Tarsi long and tapering with very mild subapical humps. Length of tarsus I, 1·36 mm., length of tarsus IV, 1·25 mm., metatarsus IV, 0·86 mm.

Remarks. A female specimen of *I. auritulus*, from a thrush at Oroya, Peru, collected on 7 March 1913 by E. H. Townsend, in the Nuttall Collection, 2518 (British Museum of Natural History), agrees with the specimen from *Caloptes rupicola* as far as the scutum, auriculae and ventral view of the basis are concerned. It differs from it, however, in having more stongly developed coxal spurs and the postero-internal coxal saliences being more emphasized.

These specimens differ from the types, electotypes and from the North American species, as described by Cooley & Kohls, in auricular pattern and hypostomal dentition.

(*d*) *Specimens from Guatemala*

The female specimen described below was obtained from a bird (?) at Sierra de Las Minas, Dept. Progresso, Guatemala, 7 February 1942, and is deposited at the Rocky Mountain Laboratory, Montana (U.S.P.H.S. no. 32153).

Female (Figs. 16–19):

Body. Sclerotized parts reddish brown.

Capitulum. Length 0·7 mm., width 0·58 mm. Basis about 2·4 times as broad as long; posterior margin slightly undulate near base of cornua; latter elevated, large (Fig. 16). Lateral margins broadly arcuate. Porose areas large, separated from each other by an interval about equal to the width of one of them. Palpal article I large, triangular dorsally, the horn-like anterior projection attenuated and dorsally directed (Fig. 16*a*), meso-dorsal edge slightly salient (Fig. 16*a*). Articles II and III clavate, combined length 0·38 mm. Sutural line faint. Basis in ventral view smooth, well constricted at about mid-length. Auriculae as large, heavy horns (Fig. 17). Postero-lateral and posterior margins rounded.

Hypostome. Mutilated.

Scutum. Length 1·23 mm., width 0·97 mm. Elongate-oval (Fig. 18), broadly rounded behind with greatest width at about mid-length, thence antero-lateral margins straight and converging on reduced humeral angles. Surface smooth, shallow punctations of moderate size, few, concentrated on the elevated lateral fields, and anteriorly and posteriorly on the median field. A faint indication of lateral carinae. Hairs very few and fine. Cervical grooves broad anteriorly and form shallow, narrow valleys posteriorly, attain postero-lateral borders on one side but not on the other in the available specimen.

Legs. Moderate in size, smooth, shining with few hairs. Tarsi long and tapering with mild subapical humps.

Coxae (Fig. 19). Convex, smooth, shining, with few long hairs. Internal spur on coxa I short, broad and heavy, those on II and III distinct but small; absent on IV. External spurs on all coxae large, those on II–IV not differing markedly in size. Trochantal spurs present, distinct but distinctly smaller than external coxal spurs.

Remarks. The horn-like protrusion of palpal article I, in the types, electotype and in North American specimens, is much as figured in Fig. 1, i.e. it is not attenuated or drawn out dorsally. This specimen from Guatemala, on the contrary, has a more attenuated and more dorsally directed horn. A similar feature was commented on in another Guatemalan specimen collected from *Oreophasis* sp., Volcan Tajumulco, San Marcos (23 February 1934) [Rocky Mountain Laboratory collection, 12034], by Cooley & Kohls (1945).

More material from this region should be interesting to examine.

(*e*) *North American specimens*

The characters of the North American specimens examined show substantial agreement with the type and electotype specimens, except for coloration and the shape of the scutum. Attention to differences in the shape of the scutum were first noted by Nuttall (1916). He observed that the scuta of the Canadian specimens were broader than those of the types, the coxae were slightly broader and the legs somewhat longer than in the type. The correctness of these observations I corroborate, and I also believe the individual segments of the leg to be broader. Subsequently, Cooley & Kohls (1945) described this species from North America, and there is nothing to add except that the trochantal spurs are small and distinct. Doubtless owing to intraspecific variation there will be discrepancies in some of the measurements; thus Nuttall (1916) gives the measurements of the scutum from the Canadian specimen as 1·4 × 1·2 mm., Cooley & Kohls (1945) as 1·32 × 1·02 mm., Gregson (1956) states that it is as broad as long, and my own observations show a variation from 1·3 × 1·14 to 1·28 × 1·24 mm. These differences are well within the possible range of variability for, as shown elsewhere (Arthur, 1949), the range in scutal size of females of *I. ricinus* is from 1·18 × 1·18 mm. to 1·51 × 1·44 mm.

(*f*) Ixodes auritulus *from Snares Island* (*New Zealand*)

This material was originally described by Dumbleton (1953) from a single male, one female and one nymph collected in the nest material of the common diving petrel (*Pelecanoides urinatrix*) on Snares Island, December 1947.

Female (Figs. 20–23):

Body. Colour brown. Broadly rounded behind, narrowing appreciably to the insertion of basis capituli. Legs thin, relatively long, with two distal segments of fourth pair of legs extending beyond posterior extremity of body.

Capitulum. Length 0·51 mm., width 0·41 mm. Basis 2–3 times as long as broad, surface flat except for an oval depression between the porose areas, lateral surfaces elevated. Posterior margin straight, lateral margins arcuate; cornua subtriangular with rounded apices, broader basally than long and slightly elevated postero-dorsally. Porose areas elongate-oval, superficial. Palpi club-shaped as figured (Fig. 20), combined lengths of articles II and III 0·27 mm., sutural line faint; article I large, attenuated and dorsally directed anterior horn, meso-dorsal surface with antero-posterior flange as shown in Figs. 20 and 20*a*. Basis in ventral view flat and smooth except for slight depression at posterior origin of auriculae. Latter as broad, heavy spurs with rounded apices (Fig. 21).

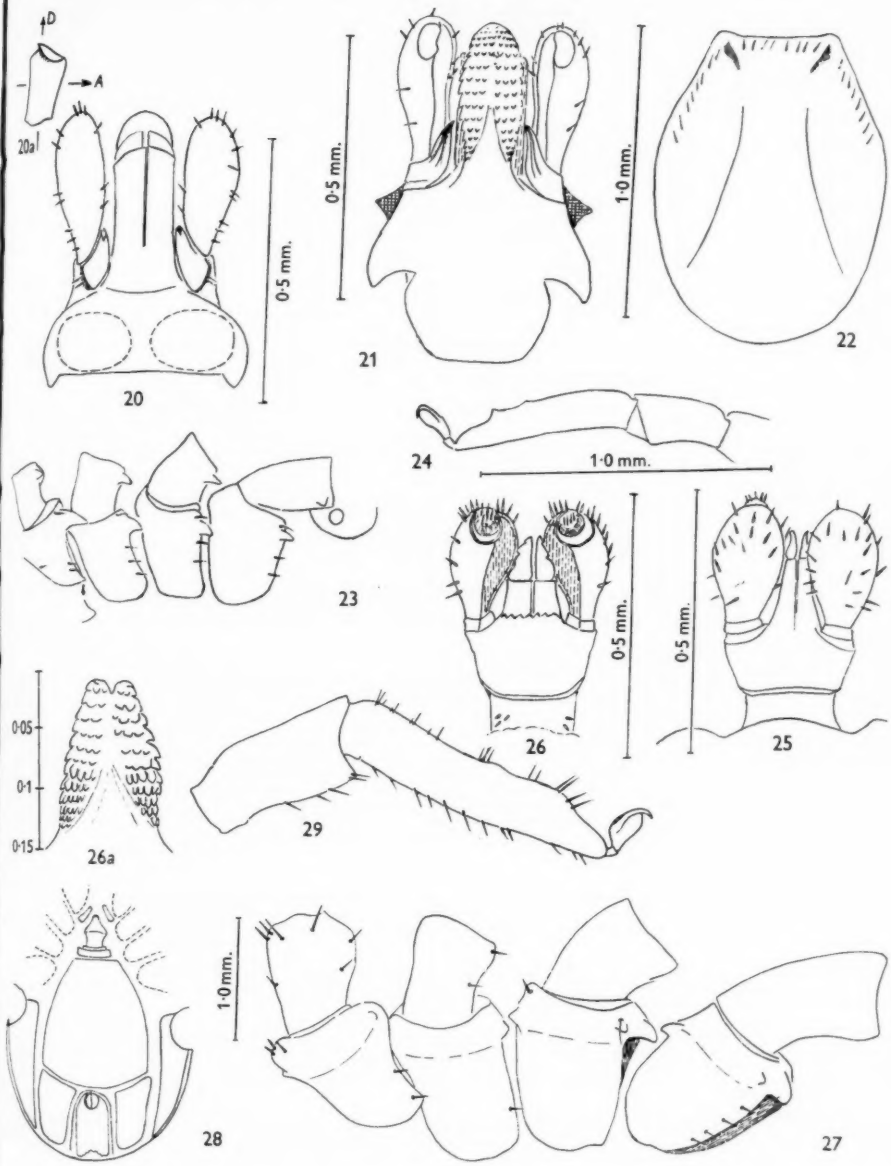
Hypostome. Wide and broadly rounded apically. Behind small corona dentition on right side as three rows 5/5, six rows 4/4 (distal two rows with additional smooth teeth mesially), two rows of 3/3 files, two rows of 2/2 files and one row of 1/1 files; on the left side one row of 5/5 files, seven rows of 4/4 files, two rows of 3/3 files, two rows of 2/2 files, one row of 1/1 files. Length 0·28 mm. (Fig. 21).

Scutum. Length 1·1 mm., width 0·8 mm. Elongate-oval, broadly rounded behind, flattened antero-laterally, greatest width about mid-length. Humeral angles much reduced. Cervical grooves long, shallow depressions, converging at first then diverging and almost reaching the postero-lateral edge. Surface convex between cervical grooves, flat in the lateral fields and gently sloping peripherally. Punctations few, large, shallow, sometimes confluent. Hairs short, spinose, concentrated anteriorly and antero-laterally, of equal length to those on the post scutum (Fig. 22).

Legs. Rather long and thin, with few hairs. Tarsi long and tapering with fairly well-defined subapical humps.

Coxae. Distinctly longer than broad, coxa IV a little larger than either II or I; gently convex, except for I with its steep posterior face. Internal spur on coxa I very small, almost a salience as are those on coxae II and III; absent on IV. External spur on coxa I large and heavy, those on II to IV smaller and all of similar size. Trochantal spurs present but smaller than corresponding external coxal spurs. Hairs spinose, of moderate length (being about half the length of those of *I. auritulus sensu stricto*) (Fig. 23).

Spiracular plate. Subcircular, macula slightly eccentric.



Figs. 20–29. *I. auritulus* from Snare Island, New Zealand sub-region. Figs. 20–24 female: 20, capitulum, dorsal; 20*a*, side view of palpal article 1; 21, capitulum, ventral; 22, scutum; 23, coxae and trochanters I–IV; 24, tarsus and metatarsus I. Figs. 25–29 male: 25, capitulum, dorsal; 26, capitulum, ventral; 26*a*, hypostome; 27, coxae and trochanters I–IV; 28, ventral plates; 29, tarsus and metatarsus I.

Male (Figs. 25–29).

Body. Ovate, length 2·61 mm., width 1·6 mm., colour brown. In the specimen posterior part of dorsum sunk inwards with a region of less heavy sclerotization. Slightly larger than female.

Capitulum. Length 0·38 mm., breadth behind palpal base 0·26 mm. Dorsal ridge short, curved postero-laterally, lateral margins straight and divergent to palpal bases. Surface slightly elevated, convex in front and peripherally. No cornua. Palpi short and broad, combined lengths of articles II and III 0·20 mm., greatest width of 0·13 mm. at about mid-length. Hairs spinose, of moderate length, particularly marginally and apically (Fig. 25). Basis in ventral view with broadly rounded posterior and postero-lateral margins, lateral margins almost rectilinear and divergent anteriorly. Palpal article I broader than long with strong meso-ventral ridge. Hairs less abundant ventrally (Fig. 26).

Hypostome. Broad proximally, narrowing distally where it is strongly indented. Crenulations for just over distal half, giving way to six rows of blunt teeth, proximally as in Fig. 26*a*. Length 0·16 mm.

Scutum. Length 1·9 mm., width 1·3 mm. Oval, with slight lobes antero-laterally on a level with coxae I—probably these lobes represent the scapulae on either side; emargination negligible. Cervical grooves faint, shallow. Lateral carinae lacking. Impunctate, smooth, shining, hairless, castaneous. Lateral groove of moderate width.

Ventral plates (Fig. 28). Length of median plate 0·9 mm., breadth across its greatest width posteriorly 0·87 mm. Adanal plate slightly wider in front than behind. Anal plate parallel sided, rounded in front. Epimeral plate subtriangular, anterior margin on a level with the front edge of the spiracular plate and tapering posteriorly on a level with posterior extremity of adanal plate. Subrectangular jugular plates internal to coxae I; pregenital plate subhexagonal, postero-lateral margins concave. Almost hairless and impunctate (Fig. 28).

Legs. Of moderate length, broad. Length of tarsus I 0·58 mm., metatarsus I 0·37 mm., tarsus I parallel to beyond Haller's organ, thence slightly upcurving distally and tapering quite abruptly to the pulvillus. Claw longer than pulvillus (Fig. 29).

Coxae. Closely set together; coxa I with marginal salience postero-internally, similar saliences on coxae II-III, absent on IV. Slight spur postero-externally on coxa I, but increasing in size from coxae II–IV (Fig. 27). Antero-external spurs on coxae II–IV. Surfaces gently convex basally, becoming strongly tumescent distally. Hairs short, spinose.

Spiracular plate. Transversely oval, 0·22 mm. long, 0·17 mm. broad, macula almost central.

Genital orifice. On a level with second intercoxal space.

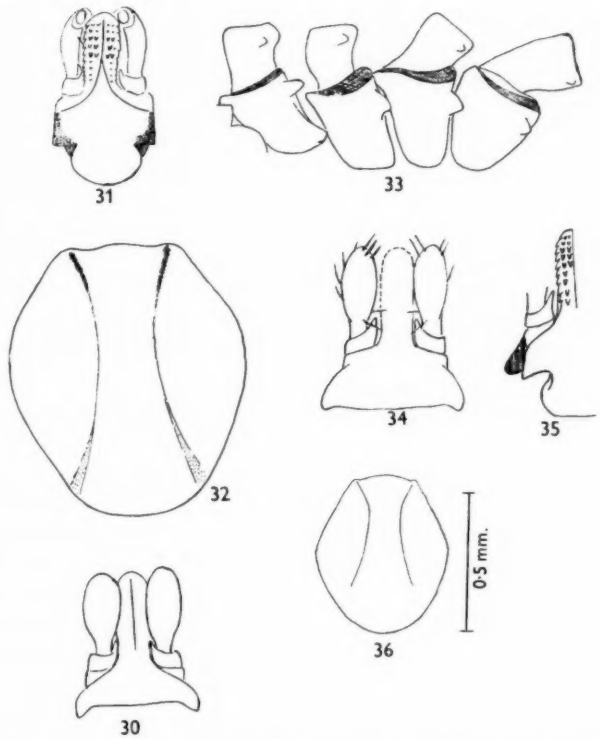
Nymph (Figs. 30–33).

Colour. Scutum and capitulum dark brown, legs and alloscutum yellow brown.

Capitulum. Length 0·27 mm., width 0·24 mm. Basis broad with cornua as

figured (Fig. 30) when seen dorsally. Palpal article I triangular dorsally and forming a horn-like protrusion directed forwards; meso-dorsal posterior edge salient. Palpi II and III club-like, combined lengths 0·15 mm. In ventral view basis long, waisted at the middle, rounded and salient behind. Postero-external angle of auriculae right-angled (Fig. 31).

Hypostome. Broadly rounded apically. Dentition as four rows of 2/2 files for proximal third, and then five rows of 3/3 files to the small apical corona.



Figs. 30–36. Figs. 30–33. Nymph from Snare Island: 30, capitulum, dorsal; 31, capitulum, ventral; 32, scutum; 33, coxae and trochanters I–IV. Figs. 34–36. *I. auritulus* nymph: 34, capitulum, dorsal; 35, capitulum, ventral (Rocky Mountain Laboratory, no. 31479); 36, scutum (G. H. F. Nuttall collection 2520 [ii] in British Museum, Natural History).

Scutum. Length 0·58 mm.; the greatest width 0·44 mm., in front of mid-length (Fig. 32). Cervical grooves reaching postero-lateral margins. Surface flat, shiny and smooth between cervical grooves, tends to be rugose laterally.

Coxae. Coxae I and II with marginal saliences postero-internally (Fig. 33). External spur on coxa I the longest, those on II–IV short and broad (not heavy as in other alleged species of *auritulus*). Trochantal spurs short and broad. Coxae longer and narrower than in other specimens of *auritulus*.

Remarks. Dumbleton (1953) states that there is little doubt that this male is

correctly associated with *Ixodes auritulus*, and Gregson (1956) accepts this view. There is no question either about the close affinity of Dumbleton's female and nymphal specimens with *I. auritulus sensu stricto*, but the occurrence of the long, relatively narrow coxae, the salient meso-dorsal ridge and the attenuated dorsal extension of the anterior horn of palpal article I contrasts with the form found in the type, electotype and other New World specimens.

Nymphae

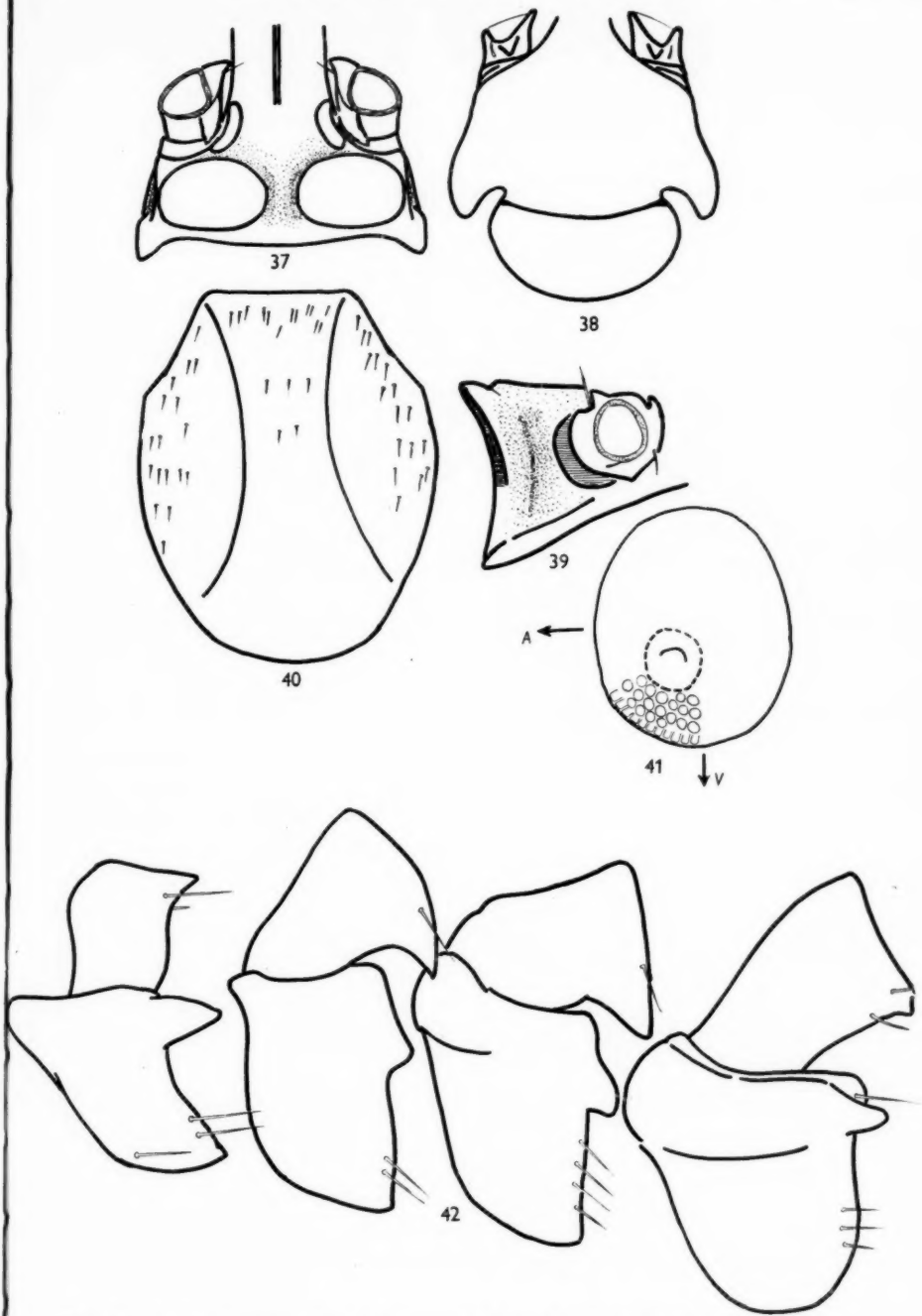
No nymphal ticks were associated with the female type specimens. In general there is substantial agreement with the description given by Cooley & Kohls, except for the Snares Island specimen (see above), and for specimens collected in Peru. In one of these specimens (collected off *Nathoprocta pentlandei* sp.sub., Cuzco, Acongate, Tinki, Peru, 20 July 1949, Rocky Mountain Laboratory collection, no. 31479) the cornua are very short and broad (Fig. 34) and the auriculae are not as retrograde (Fig. 35) as in other specimens examined. In the other Peruvian specimen (collected off pipit, Lunin, Peru, 19 March 1943, C. H. T. Townsend *leg.*, G. H. F. Nuttall collection, no. 2520 [ii]) the scutum is elongate-oval, measuring 0·58 mm. long and 0·44 mm. broad (Fig. 36) and the auriculae again are not strongly retrograde. Possibly these differences are intraspecific, but in view of what we have observed in females from the same region (see earlier) an examination of large numbers of alleged specimens of *I. auritulus* from this region might prove to be illuminating.

IXODES PERCAVATUS GROUP

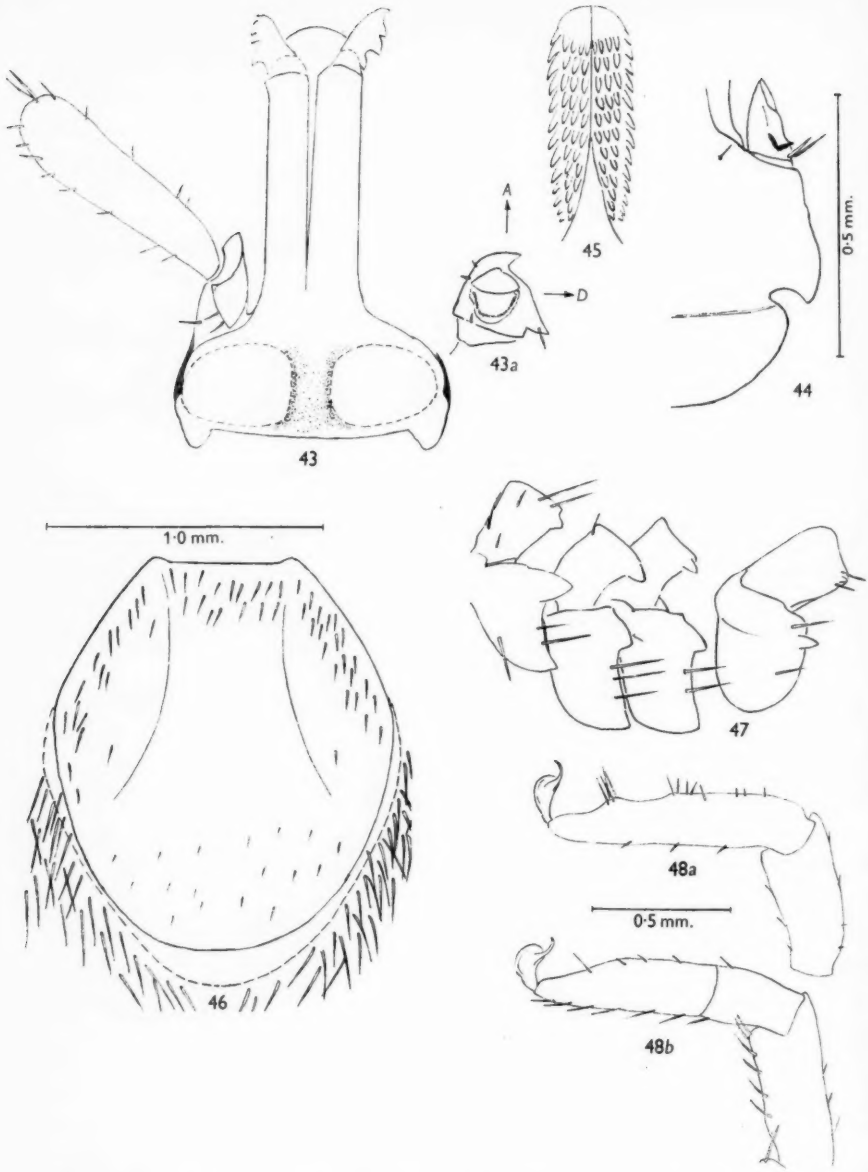
I. percavatus s.str. (Figs. 37–48*b*) based on type material (four dried, badly mutilated, gorged females, from Nightingale Island, of the Tristan da Cunha group (west of the Cape of Good Hope), British Museum, Natural History), and the electotype from *Diomedea chlororhynchus*, Nightingale Island, of the Tristan da Cunha group, M. K. Rowan *leg.*, 9 April 1950. Deposited in Department of Entomology, South African Institute for Medical Research, Johannesburg, South Africa.

Body. Colour dark brown, alloscutum abundantly covered with long spinose hairs.

Capitulum. Length 0·79 mm., width 0·55 mm. Basis broad, somewhat undulate with distinct depression between porose areas; posterior margin weakly curved, salient, little lower than the large cornua (Figs. 37, 43). Porose areas very large, oblong with definite edges, depressed, separated on the median line. Palpal article I very large, with attenuated dorsally directed horn-like protrusion; meso-dorsal margin prolonged dorsally to form a strong spur, subtended by one long and one short hair. Articles II and III club-shaped when viewed dorsally bearing both long and short spinose hairs; sutural line between articles II and III faint, combined length 0·49 mm. Basis in ventral view (Figs. 38, 44) smooth, constricted behind midlength, rounded and salient posteriorly. Distinct transverse groove behind constriction. Auriculae large, heavy and retrograde. Ventral spur on article I.



Figs. 37–42. *I. percavatus* female (Neumann's 'types'). 37, basis, dorsal; 38, basis, ventral; 39, basis, lateral; 40, scutum; 41, spiracle; 42, coxae and trochanters I–IV.



Figs. 43–48. *I. percavatus* female. Electotype. 43, capitulum, dorsal; 43*a*, side view of palpal article I, *A*—anterior, *D*—dorsal; 44, basis, ventral; 45, hypostome; 46, scutum, variation in shape indicated by broken line; 47, coxae and trochanters I–IV; 48*a*, tarsus and metatarsus I; 48*b*, tarsus and metatarsus IV.

Hypostome. Spatulate and broadly rounded at the apex (Fig. 45). Dentition behind a corona as one row of 5/5, six rows of 4/4 for over half its length, thence to the base by three rows of 3/3, and three rows of 2/2. Denticles fine and sharp, those in the lateral files a little longer. Length 0·45 mm.

Scutum. Length 1·37 mm., width 1·24 mm. Suboval to lozenge-shaped (Figs. 40, 46), broadly rounded behind, flattened on antero-lateral sides, widest at or slightly in front of mid-length. Humeral angles much reduced. Surface in two of the specimens undulate, finely punctate, punctations most abundant peripherally. Spinose hairs lateral to cervical grooves and across anterior margin of moderate length (about $\frac{1}{3}$ length of those on alloscutum); fine short hairs in posterior region between cervical grooves. Cervical grooves as distinct broad depressions, first converging and then diverging and fading out without reaching postero-lateral margins. No lateral carinae.

Legs. Moderate in size, smooth, shining with good cover of spinose hairs. Tarsi subparallel proximally (Figs. 48*a*, *b*) with mild subapical humps. Length of tarsus I, 0·97 mm., metatarsus I, 0·62 mm. Length of tarsus IV, 0·99 mm., metatarsus IV, 0·72 mm.

Coxae (Figs. 42, 47). Convex, smooth, with few long spinose hairs. Internal spurs on coxae I–III barely more than saliences. External spur on coxa I long and heavy, those on II and III short and broad, that on coxa IV longer and narrower. Trochantal spurs distinct and of variable length (cf. Figs. 42, 47). Distinct dorsal trochantal spur present.

Genital opening. On a level with coxae III.

Anal groove. Rounded in front of anus, sides straight behind anus and tending to converge slightly.

Spiracular plate. Large, transversely ovate, surface convex. Macula eccentric on ventral side. Goblets numerous.

Ixodes zumpti sp.nov. (Figs. 49–54*b*)

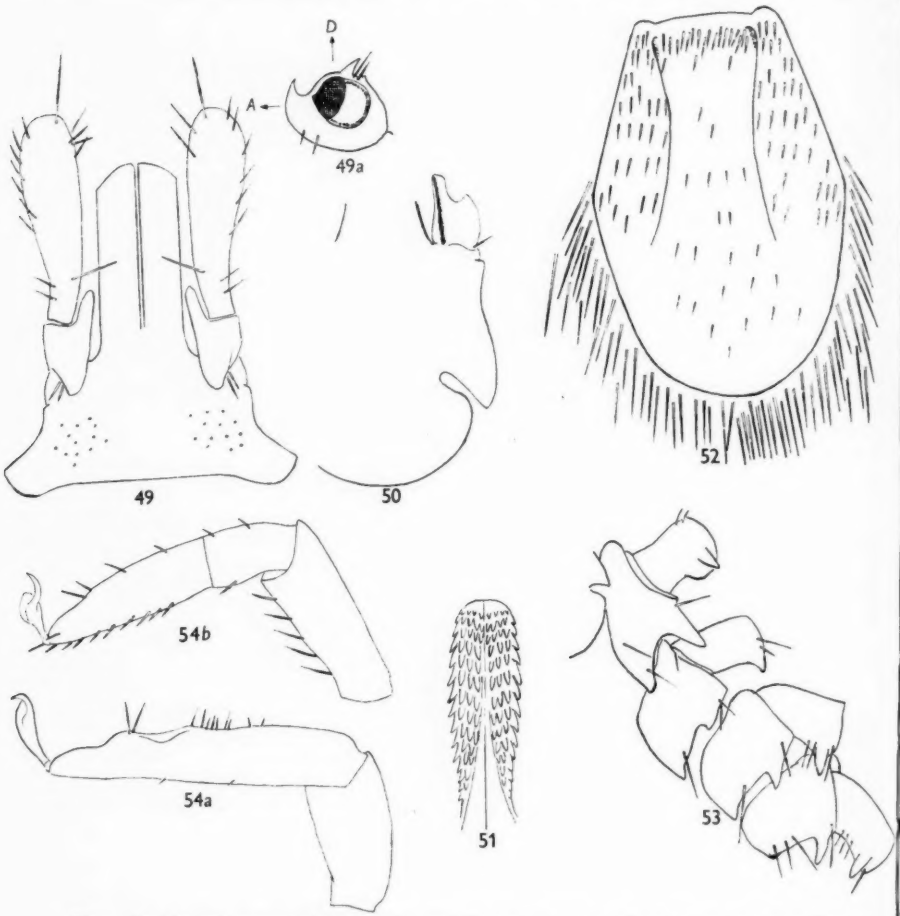
Holotype. Partially fed female, collected off *Puffinus gravis*, Nightingale Island, Tristan da Cunha. M. K. Rowan *leg.*, 4 May 1949. Deposited in the Department of Entomology, South African Institute for Medical Research, Johannesburg, South Africa.

Paratypes. One partially fed female, collected off *Diomedea chlororhynchus*, Nightingale Island, Tristan da Cunha. M. K. Rowan *leg.*, 31 March 1950. One partially fed female, collected off *Phebetus fusca*, Nightingale Island, Tristan da Cunha, M. K. Rowan *leg.*, 5 April 1950.

Female

Body. Partially engorged specimen elongate-oval. Distinct marginal fold. Scutal hairs spinose, strong. Post-scutal hairs about $2\frac{1}{2}$–3 times as long as scutal hairs, spinose postero-lateral to the scutum, less spinose and less abundant immediately behind the scutum, finer posteriorly. Shorter and finer hairs along the marginal fold. Colour dark or reddish brown.

Capitulum. Length 0·74 mm., width 0·57 mm. Basis broad, in general smooth; posterior margin straight, salient and a little lower than cornua. Latter large, broad and virtually forming elevated postero-lateral flanges (Fig. 49). Porose areas small, superficial, ill-defined, consisting of few, widely separated small 'pores'. Palpal article I very large, with horn-like protrusion directed anteriorly, horn attenuated and dorsally directed (Fig. 49*a*), meso-dorsally flanged to form prominent meso-dorsal spur subtended by one long and one short hair. Articles II



Figs. 49–54*b*. *I. zumpti* female. 49, capitulum, dorsal; 49*a*, palpal article I side view; 50, basis, ventral; 51, hypostome; 52, scutum; 53, coxae and trochanters I–IV; 54*a*, tarsus and metatarsus I; 54*b*, tarsus and metatarsus IV.

and III long, clavate when viewed dorsally, combined lengths 0·41 mm. Sutural line faint. Basis in ventral view smooth, constricted at the middle, rounded and salient behind. No transverse groove. Auriculae as large heavy horns, but not as broad as in *I. percavatus* (Fig. 50).

Hypostome. Spatulate, rounded apically. Dentition from apex to base as two rows of 5/5, six rows of 4/4 (extending proximal of mid-length), three rows of 3/3 and two rows of 2/2. Denticles fine and sharp, those in lateral files a little longer. Length 0·42 mm. (Fig. 51).

Scutum. Length 1·39–1·42 mm., breadth 0·94–0·95 mm. Elongate-oval, broadly rounded behind, antero-lateral margins flattened (Fig. 54), widest at about mid-length. Humeral angles much reduced. Surface smooth, punctations large, deep, uniformly distributed, moderate in number. Spinose hairs of moderate length, particularly dense in the anterior half beyond the cervical grooves, and as a dense patch anteriorly between the cervical grooves; between the latter in the median and posterior fields hairs finer and shorter. Cervical grooves distinct as broad depressions, very deep fossa anteriorly first converging, then diverging and fading out just beyond mid-length. Lateral carinae absent.

Legs. Longer than in *I. percavatus.* Internal spur on coxa I relatively broad and heavy, those on II and III as short distinct spurs, distinct marginal salience on IV. External spur on coxa I large and heavy, that on II flattened, arising about one-third to one-half the coxal length from the distal extremity, that on coxa III longer and recurved, the spur on IV narrower and as long as that on III. Trochantal spurs on all legs, those on III and IV particularly well developed and as long as or longer than the corresponding external coxal spurs (Fig. 53). Tarsus I with moderate subapical hump (Fig. 54*a*, *b*). Length of tarsus I, 1·15 mm., metatarsus I, 0·6 mm. Length of tarsus IV, 1·0 mm., metatarsus IV, 0·73 mm.

Spiracular plate. Transversely ovate, goblets small and numerous.

Genital aperture. Between coxae III.

Related species. I. zumpti has undoubted affinities with *I. percavatus,* but its occurrence on three distinct hosts at different times leads me to believe that it is a valid species. It differs from *I. percavatus* in the form of the cornua, the poor differentiation of the porose areas, the absence of the transverse groove, the smooth, more heavily punctate scutum as well as the shape of the latter.

Ixodes pterodromae sp.nov. (Figs. 55–62*b*)

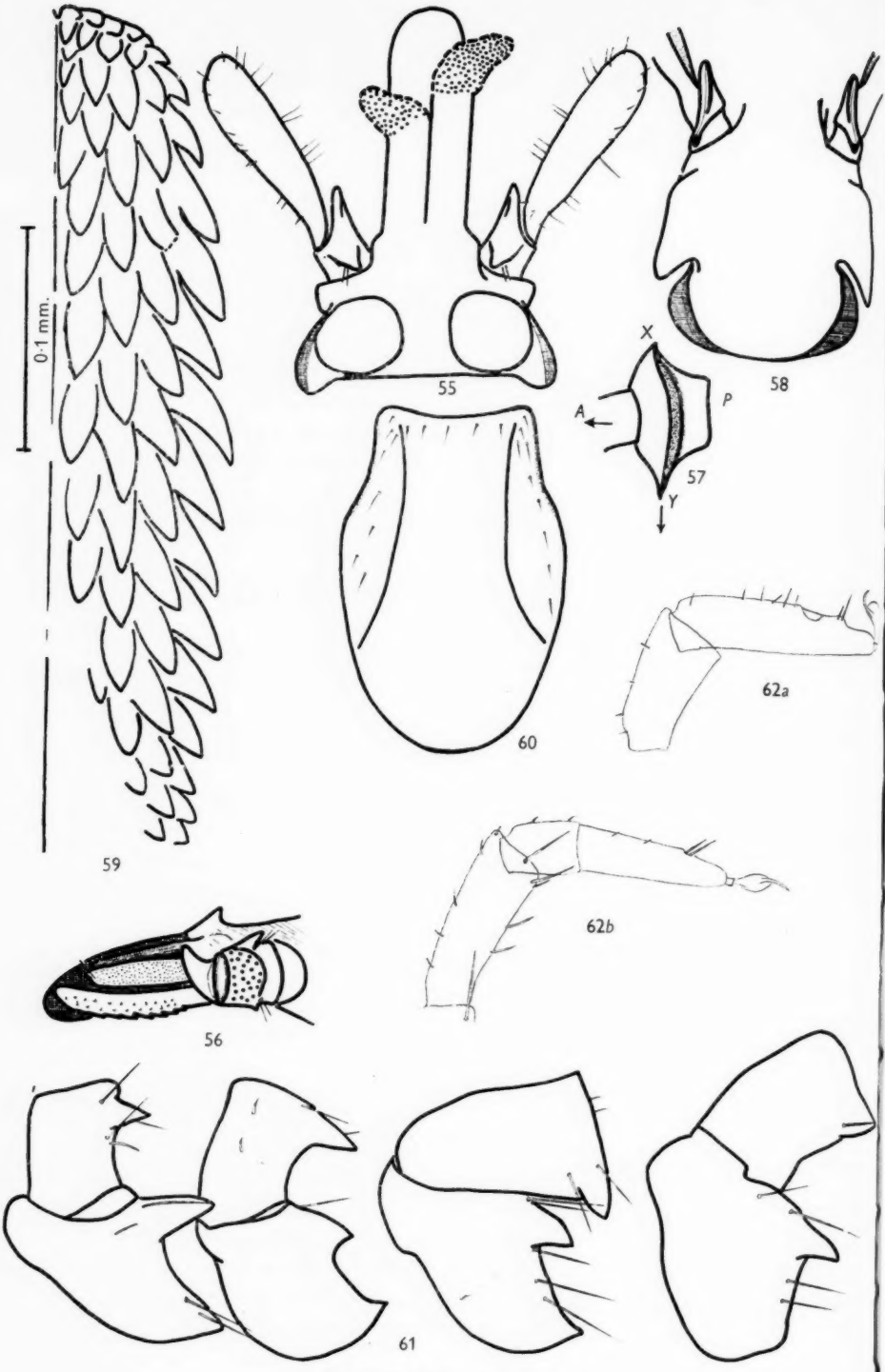
Holotype. Female from Marion Island, collected from *Pterodroma brevirostris,* 18 November 1951. R. W. Rand *leg.* Deposited in the Onderstepoort Research Station.

Paratype. Female from Marion Island, collected from *Pterodroma mollis,* 18 November 1951. R. W. Rand *leg.*

Female

Body. Heavily pigmented, alloscutal and scutal hairs spinose, former twice as long as latter.

Capitulum. Length 0·79 mm., maximum width 0·54 mm. Posterior margin straight, salient. Cornua rounded, shorter than their basal breadth (slightly foreshortened in Fig. 55), postero-laterally directed and elevated above the posterior margin; antero-lateral margins continuous with lateral margin and curving strongly to the palpal insertion. Surface generally flattened, with large



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almost circular porose areas, separated from each other by about half the diameter of one of them (Fig. 55). Palpal articles 2 and 3 clavate when seen dorsally, hairs of moderate length; combined length of articles 2 and 3, 0·47 mm.; suture lines between articles 2 and 3 indistinct. Palpal article 1 very large, with anteriorly protruding horn, which is attenuated and strongly curved dorsally (Fig. 56); strong spur meso-dorsally, subtended at its base by two hairs. Basis in ventral view broadest across auriculae (Fig. 58), latter as broad spurs with almost straight lateral margins. Posterior margin broadly rounded or subangular, sides steep; mesial face of articles 2 and 3 almost flat, hairs slightly longer on ventral than on dorsal edges of palps, small spur meso-ventrally on article I.

Hypostome. Spatulate, narrowing proximally, broadly rounded apically; dentition from apex to base as two rows of 5/5, eight to nine rows of 4/4, two to three rows of 3/3, one row of 2/2; teeth almost equal in all rows, generally narrow and backwardly directed (Fig. 59).

Scutum (Fig. 60). Length 1·35 mm., breadth 0·9 mm. Elongate-oval, broadest about mid-length. Antero-lateral borders slightly concave, lateral and postero-lateral borders gently convex terminating in broadly rounded posterior edge. Surface glossy brown anteriorly and peripherally, otherwise yellowish brown. Cervical grooves long, broad, shallow, diverging slightly to postero-lateral border, less heavily pigmented than the rest of the scutum. Spinose hairs of moderate length, abundant antero-laterally and anteriorly. Lateral carinae lacking. Punctations of two kinds, one being large and the other small, but both shallow, unequally spaced and frequently confluent.

Legs. Broad and of moderate length. Tarsi long, tapering with moderate subapical hump. Length of tarsus I, 0·88 mm., metatarsus I, 0·72 mm. Length of tarsus IV, 0·89 mm., metatarsus IV, 0·59 mm. (Fig. 62*a*, *b*).

Coxae (Fig. 61). Brown, smooth, glossy, with few long, fine hairs along posterior margins. Internal spur on coxa I short, broad, heavy; those on II and III distinct and pointed, that on IV a marginal salience. External spurs on all coxae, very large on coxa I, shorter and broader on II, and increasingly large on III and IV. Trochantal spurs very strong, those on II, III and IV as long as or longer than external coxal spurs. Large dorsal spur on trochanter I (Fig. 57), smaller one on trochanter II.

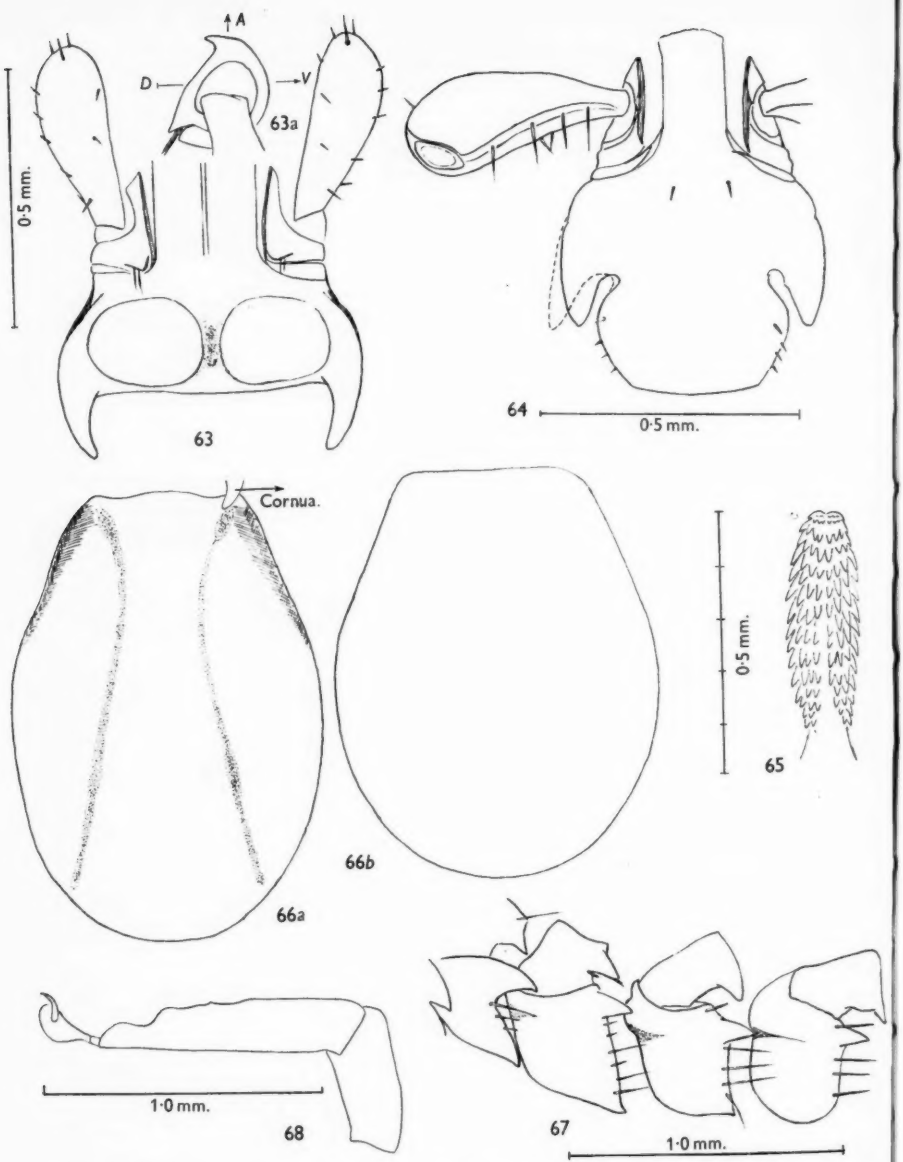
Spiracular plate. Whitish, slightly transversely oval. Goblets numerous and small. Macula a little eccentric on the antero-dorsal side.

Genital orifice. Between coxae III.

Related species. Like *I. zumpti*, this new species has an undoubted affinity with *I. percavatus*. It is readily separated from these two species on the character of the cornua, the porose areas and the coxal armature.

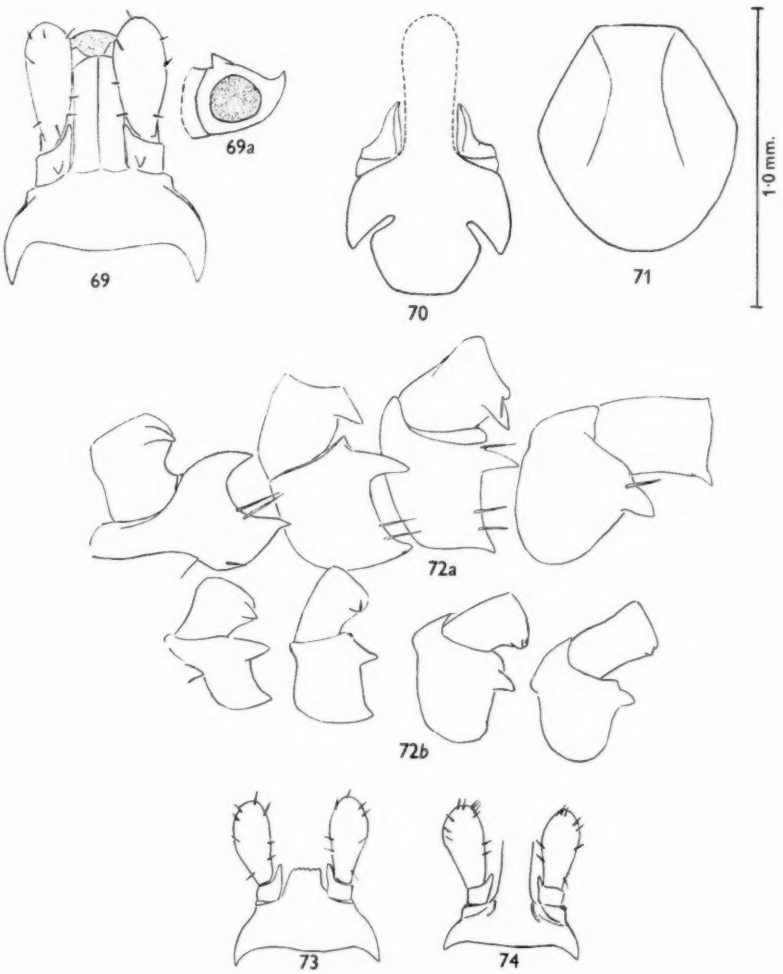
Two badly mutilated females of what appears to be *I. zumpti* along with one

Figs. 55–62*b*. *I. pterodromae* female. 55, capitulum, dorsal; 56, capitulum, side view after removal of palpal articles II–IV; 57, side view of trochanter I to show dorsal process (*X*) and ventral spur (*Y*); 58, basis, in ventral view; 59, hypostome; 60, scutum; 61, coxae and trochanters I–IV; 62*a*, tarsus and metatarsus I; 62*b*, tarsus and metatarsus IV.



Figs. 63–68. *I. cornuae* female: 63, capitulum, dorsal; 63*a*, side view of palpal article I, *A*—anterior, *D*—dorsal, *V*—ventral; 64, capitulum, ventral; 65, hypostome; 66*a*, scutum as shown in three of the four specimens examined; 66*b*, outline of scutum of the fourth specimen examined; 67, coxae and trochanters I–IV; 68, tarsus and metatarsus I.

female of *Ceratixodes uriae* White and eight males of *I. kerguelenensis* André and Colas-Belcour were collected from the burrows of Prions and diving petrels at West Bay, Heard Island (A.N.A.R.E. collection 720). Accordingly, the females of *I. zumpti* may be the females of *I. kerguelenensis*. More convincing evidence



Figs. 69–72*a*. *I. cornuae* nymph: 69, capitulum, dorsal; 69*a*, palpal article I, lateral view; 70, capitulum in ventral view; 71, scutum; 72*a*, coxae and trochanters I–IV; 72*b*, coxae and trochanters of nymph of *I. auritulus* from North America. Figs. 72*a* and 72*b* are drawn to the same scale for comparison. Figs. 73–74. Capitula of nymphs of *I. auritulus* from Wrangell Island, Alaska (Fig. 73 from Rocky Mountain Laboratory, Montana, no. 27286) and from Junin, Peru (Fig. 74, G. H. F. Nuttall collection, no. 2520 [ii] deposited in British Museum, Natural History). Illustrated for comparison with other nymphae referred to in this paper.

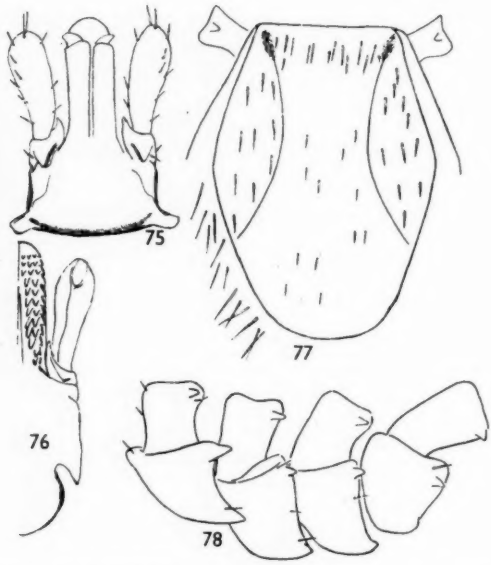
of this relationship will, however, be necessary before finally synonymizing *zumpti* with *kerguelenensis*. The male of the latter species is redescribed elsewhere (Arthur, 1960).

Nymphs from Nightingale Island

Source of material. (i) Seven nymphae, off *Diomedea chlororhynchus*, Nightingale Island, Tristan da Cunha. M. Rowan *leg.* Deposited in Medical Research Department, Johannesburg, S. Africa; (ii) one nymph, Skua chick (*Skua lonnbergi*), A.N.A.R.E. expedition, Macey Cove, Heard Island, 15 February 1955, G. M. Budd *leg.*

Sclerotized parts yellow to brown; body densely covered with bristle-like hairs.

Capitulum. Broad, flat except posteriorly (shaded in Fig. 75) and postero-laterally which are steeply elevated. Length 0·4 mm., breadth across cornua



Figs. 75–78. Nymph from Nightingale Island. 75, capitulum, dorsal; 76, capitulum, ventral; 77, scutum; 78, coxae I–IV.

0·33 mm. Cornua broad, virtually flanges directed postero-laterally. Palpi short, broader distally, with lateral profile lines weakly concave, inner profile convex. Article I, viewed dorsally, with short, upcurved dorsal horn directed anteriorly, and strong meso-dorsal spur. Sutural lines between articles II and III faint, their combined lengths 0·2 mm. In ventral view, basis constricted at the middle, wider in front than behind, with posterior margin rounded. Auriculae present as retrograde spurs, whose lateral margins are almost rectilinear. Palpi flattened on their inner faces, hairs few in number.

Hypostome. Rounded apically. Denticles arranged 4/4 distally, but for greater part of length as 3/3. Length about 0·23 mm. (Fig. 76).

Scutum. Length 0·62 mm., width 0·45 mm. Greatest width at or slightly in front of mid-length, antero-lateral margins almost straight, posterior margin broadly rounded. Cervical grooves as broad valleys extending back to postero-lateral margins (Fig. 77). Surface smooth, shiny, with few moderately large punctations. Hairs spinose, being particularly abundant anteriorly and beyond the cervical grooves. No lateral carinae.

Coxae (Fig. 78). Surfaces slightly convex, smooth, shining and with punctations very fine or absent. Internal spurs distinct on coxae I–III, sometimes a marginal salience on IV. External spur on coxa I stout and strong, those on II–IV of somewhat similar size, but all well developed. Trochanter spurs on II–IV approaching those on postero-external margins of coxae in respect of size. Tarsi with slight subapical humps.

Remarks. Anastos (1954) described the female, nymph and larva of *I. percavatus* based on '2 females and 29 nymphs off an albatross (*Diomedea chlororhynchus*) from Nightingale Island and 4 larvae off the same host from Tristan da Cunha Island, and 1 larva on a hermit thrush, *Nesocichla eremita gordoni* (Starchies) from Inaccessible Island'. The nymphs described in the present paper do not agree with those described as *I. percavatus* by Anastos and, indeed, in many features agree more closely with the adults of *I. zumpti*. In view of their uncertain position I propose to leave these nymphae unnamed until such time as we are able to relate them more confidently to their adult stages.

Ixodes cornuae sp.nov. (Figs. 63–72*a*)

The following description is based on (i) three ticks 'Parasites trouves sur une espèce de Caille, Equateur, Dr Rivet *leg.* 1901' (in the Paris Museum of Natural History) and (ii) a single well-gorged specimen taken by the 'Baie Orange Mission du Cap Horn, 1882–8, Dr Hyades and Dr Sauvinet, no. 3063–1885' (Paris Museum of Natural History). Both sets of specimens were identified as *I. auritulus* by Neumann, but the presence of the strong meso-dorsal spur on article I (Fig. 63, *A*) precludes it from inclusion in the *auritulus* group, and it obviously falls into the *percavatus* group.

All these specimens are female ticks which are either partially or almost completely fed. The Equateur specimens measure 6 × 5 mm., 7 × 5 mm., 6·5 × 5 mm., whilst the specimen collected by Hyades and Sauvinet is 15 × 9 mm.

Female

Colour brown, elongate-oval in form.

Capitulum. Over-all length 0·8 mm., width 0·58 mm. (Fig. 63). Basis about 2·3 times as broad as long and in general smooth; posterior margin either straight or only slightly convex, salient. Lateral margins curved, dorso-ventral margin steep. Cornua slightly elevated over posterior margin, long, being about twice their basal breadths, inner margin concave, outer margin convex. Porose areas ovate, slightly depressed with a superficial reticulate appearance, separated from each other by a narrow interval which is depressed. Palpal article I very large,

triangular dorsally, with horn-like protrusion directed forwards, this horn strongly curved distally and pointed (Fig. 63, *A*); postero-mesal edge flanged and elevated so that when seen laterally it appears as a dorsal spur. Articles II and III lacking distinct sutural line, clavate in form, combined lengths 0·35 mm. Hairs short, except near apex and most numerous peripherally. Basis in ventral view (Fig. 64) smooth, slightly convex and broadest across auriculae. Posterior margin almost flat, postero-lateral margins curved, strongly constricted at mid-length behind origin of auriculae. Latter as large, heavy horns extending well back (Fig. 64). Spinose hairs postero-laterally and a pair of fine hairs behind hypostomal base. Palpal article I keeled along its inner ventral edge, inner face flat or at most only slightly convex.

Hypostome (Fig. 65). Apex rounded with slight median indentation. Teeth arranged in fourteen rows of 4/4 files, with median unarmed area proximally. Lateral teeth long, narrow, pointed. Length 0·45 mm.

Scutum (Figs. 66*a*, *b*). Some variation in size and shape, measuring 1·45 × 1·12, 1·64 × 1·09, 1·51 × 1·09, 1·5 × 1·10 mm., longer than broad. Broadly rounded behind and narrowing anteriorly at about one-third of its length, flattened on the antero-lateral sides and widest at or in front of mid-length. Humeral angles much reduced. Cervical grooves distinct as broad linear depressions, arising anteriorly from broader depressions, first converging and then diverging to the postero-lateral border. Surface beyond the cervical grooves rises gradually to elevations in the positions of the lateral carinae. Hairs fine, of moderate length and most abundant anteriorly and antero-laterally. Surface smooth. Punctations of moderate size, shallow and widely separated from one another.

Legs. Moderate in size, smooth, shining, with few hairs. Tarsi long and tapering with moderate subapical humps. Length of tarsus I, 1·0 mm., metatarsus I, 0·59 mm. Length of tarsus IV, 0·91 mm., metatarsus IV, 0·64 mm. (Fig. 68).

Coxae. Convex, smooth, shining, with depressions as indicated by stippling in Fig. 67. External spurs on all coxae long and broad, those on II–IV of similar size. Internal spur on coxa I heavy, tapering and pointed (in one specimen directed to the mid-line), on coxae II and III they are distinct and prominent. Trochanter spurs on I–IV, heavy, broad basally and narrowing apically, almost as large as external coxal spurs.

Spiracular plate. Almost circular, macula eccentric on antero-ventral side. Goblets moderate in size and number.

Genital aperture. Between coxae III.

Whilst it is by no means certain, the nymphs collected by Dr Hyades and Sauvinet during the 'Baie Orange Mission du Cap Horn, 1882–3' probably belong to this new species, although determined as *I. auritulus* by Neumann (Paris Museum Natural History, 3067–1885). I base this supposition on the presence of the meso-dorsal spur on article I and the long, tapering, almost parallel cornua. A full description of the nymph is given below.

Colour straw yellow.

Capitulum. Length 0·5 mm., width 0·39 mm. (Fig. 69). Basis broad with cornua long, tapering and almost parallel, elevated above posterior margin which is convex.

Surface smooth, impunctate. Palpal article I very large, hexagonal dorsally, the anterior horn attenuated and dorsally directed; a prominent projection on meso-dorsal surface when viewed laterally (Fig. 69*a*). Palpi club-shaped when viewed dorsally, combined lengths of articles II and III 0·24 mm. In ventral view basis is long, strongly constricted at mid-length, posterior margin straight, sides arcuate. Auriculae as large, triangular projections which are a little divergent (Fig. 70).

Hypostome. Broadly rounded apically, spatulate in outline. Dentition behind a small corona, of one row 4/4 teeth, eleven rows 3/3 teeth, one row 2/2 teeth. Length 0·26 mm.

Scutum. Length 0·75 mm., width 0·69 mm. Suboval. Cervical grooves hour-glass shaped but not reaching postero-lateral edge.

Legs. Moderate in size, smooth, shining, with few hairs. Coxae almost flat, with few hairs. Internal spur on coxa I strong and tapering; those on II and III distinct; absent on IV. External spurs on all coxae large and heavy. Trochantal spurs on all legs only slightly weaker than the external coxal spurs (Fig. 72*a*).

Spiracular plate. Goblets relatively larger and fewer in number than in the female.

Related species. I. cornuae belongs to the *percavatus* group of ticks and is distinguished from *I. percavatus*, *I. zumpti* and *I. pterodromae* on the cornual pattern.

THE UNANSWERED PROBLEMS OF SPECIATION OF BIRD TICKS

As a result of this broad survey it is evident that far more collecting of ticks on sea birds is necessary to establish the true systematic status of these parasites. This review prompts us to ask, finally, whether the differences observed are real in as far as speciation is concerned, or do they fall within the normal limits of intra-specific variation? Secondly, whether such differences, if they are specific or subspecific, have arisen as a result of geographical isolation in a manner parallel to their hosts? If so, have these changes been effected by mutation or by a process of continuous evolution? Thirdly, since the ultimate success of these ticks must depend on an adequate host supply, which presumably must be avian, are these parasites on an island dependent on migratory birds or are they non-selective as far as hosts are concerned? The extensive distribution of *percavatus* on sea birds suggests that this species is selective, but the North American form of *I. auritulus* occurs to a far greater extent on non-marine birds than it does on sea birds. In fact, one queries whether *I. auritulus* is a tick of sea birds at all, particularly when it is realized that the earliest records do not include the names of the hosts. Doubtless confusion in diagnosis has been responsible for our present uncertainty regarding many of these parasites and accordingly of their ecological relationships.

SUMMARY

1. *Ixodes auritulus* group of ticks and *Ixodes percavatus* group of ticks are both characterized by having the palpal article I drawn out to a horn anteriorly, but in the *percavatus* group there is a strong meso-dorsal 'spur' on this article, which is lacking in the *auritulus* group.

2. The mutilated types of *I. auritulus* and *I. percavatus* sp. *revocata* are redescribed and electotypes established for both.

3. Variations from *I. auritulus sensu stricto* are described from Peru and Guatemala, and a revised description given of the male, female and nymph from Snares Island.

4. *I. percavatus* and *I. zumpti* sp.nov. have been diagnosed from material from Nightingale Island, Tristan da Cunha. Ticks from Marion Island are identified as a new species, *I. pterodromae*. Female and nymphs from the Paris Museum originally identified by Neumann as *I. auritulus* now fall into the *percavatus* group and are named *I. cornuae* sp.nov.

Thanks are expressed to the following for the loan of specimens and literature upon which the paper was based: Professor M. André, Paris Museum; Dr G. Anastos, Maryland; Professor Brizzard, École Vétérinaire, Toulouse; the Curator of the Wellington Museum, New Zealand; Dr G. O. Evans, British Museum (Natural History); Dr Glen M. Kohls, Rocky Mountain Laboratory, Montana; Dr Gertrud Theiler, Onderstepoort Veterinary Research Station, South Africa and Dr F. Zumpt, Institute of Medical Research, Johannesburg.

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A review of some ticks (Acarina: Ixodidae) of sea birds

Part III. A re-description of the male of *Ixodes kerguelenensis* André & Colas-Belcour, 1942

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(*Received* 30 *July* 1959)

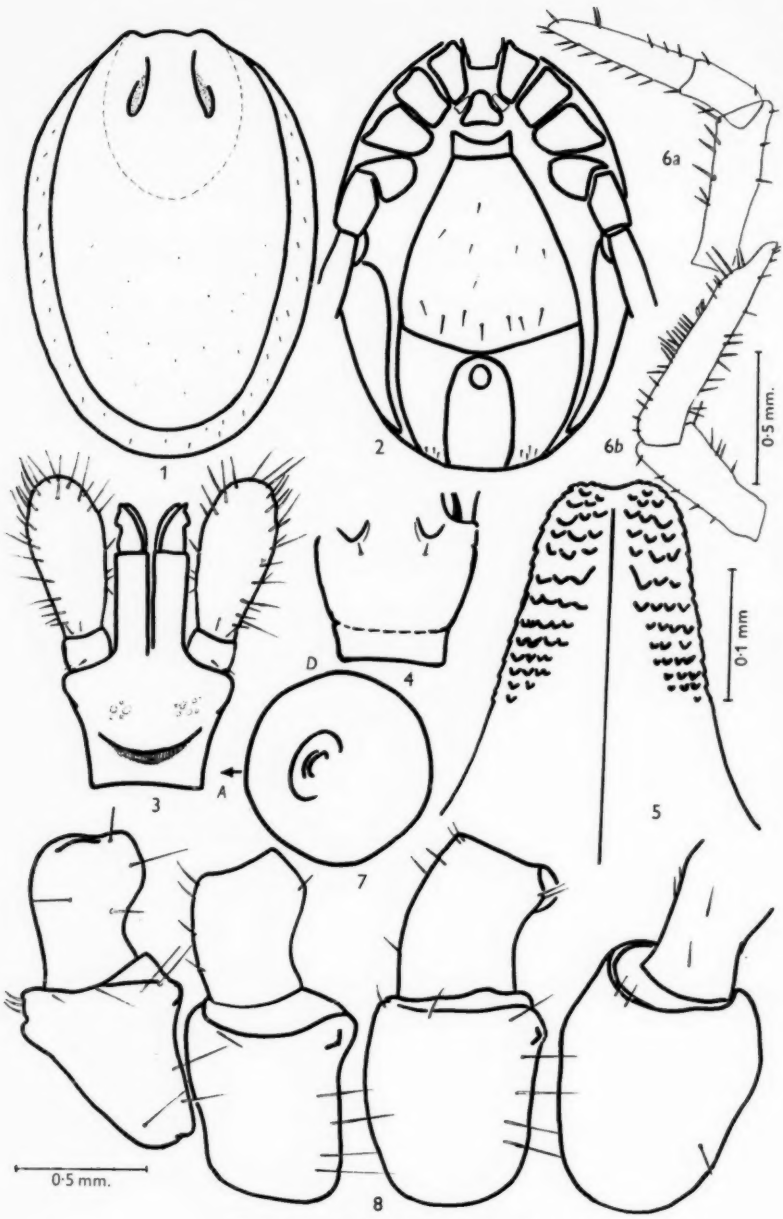
André & Colas-Belcour (1942) described the male of *Ixodes kerguelenensis* as a variety of *I. canisuga* on the basis of one specimen. Subsequently Zumpt (1952) stated that its status as a full species was unquestionable, for, compared with *I. canisuga*, the jugular plates on each side of the pregenital plate in *I. kerguelenensis* are large. The structure of Haller's organ also fully justifies this separation (Arthur, 1956). Zumpt wrote further that: 'The specimen on which the authors based *kerguelenensis* was probably fully engorged so that the ventral plates would be widely separated from each other. . . . The single male from Kerguelen is drawn as having the anal groove diverging, but this is certainly due to a distortion of the engorged tick.' Through the courtesy of Professor André I was permitted to examine the type. Distortion is not due to engorgement, as Zumpt suggests, but to the mounting of the specimen on a slide under a cover-glass.

Dr Zumpt sent me (i) a collection of eight males of *I. kerguelenensis* which were collected from the burrows of Prions and diving petrels, West Bay, Heard Island (A.N.A.R.E. collection no. 720) and two mutilated females of *I. zumpti* (?) (see Part II) and (ii) a male, Laurens Peninsula, Heard Island, 24 March 1953 (A.N.A.R.E. Acc. no. 453/A 1/1) A. M. Gwynne *leg.* The following revised description is based on this material.

Body. Size ranges from 3·2 × 2·0 mm. to 3·7 × 2·3 mm. Colour brown. Ovate and widest slightly in front of mid-length (Fig. 1).

Capitulum. Length 0·41–0·43 mm., width 0·31–0·33 mm. Basis broader than long, generally flattened in front of dorsal ridge with few scattered shallow depressions (Fig. 3). Dorsal ridge salient, curved or subangular; slope declivitous behind ridge, but gradual postero-laterally. Lateral margins almost rectilinear to palpal insertion. Cornua absent. Palpi club-shaped, short, broad, no sutural line between articles II and III; combined length of articles II and III 0·3 mm., greatest width 0·14 mm. Long spinose hairs particularly on outer margin and apex, shorter and fewer on inner margin. Basis in ventral view longer than broad, sides slightly curved and convergent posteriorly. Surface flat with slight constriction nearer to posterior margin, which is continued into a transverse groove (Fig. 4). No auriculae.

Hypostome. Broad and tapered as in Fig. 5, apex either gently curved or flat with slight median indentation. Crenulations arranged for about half the length



Figs. 1–8. *I. kerguelenensis* male

Fig. 1. Dorsal surface of opisthosoma.
Fig. 2. Ventral view of opisthosoma.
Fig. 3. Capitulum, dorsal.
Fig. 4. Basis capituli, ventral.
Fig. 5. Hypostome.
Fig. 6. *a*, Tarsus and metatarsus IV; *b*, tarsus and metatarsus I.
Fig. 7. Spiracle. *A*, anterior; *D*, dorsal.
Fig. 8. Coxae and trochanters I–IV.

from the tip as 4/4 rows, fading out obliquely at three-quarters the length. Proximal portion lacking crenulations. Length 0·28 mm.

Scutum (Fig. 1). In specimens from West Bay, Heard Island, scuta have sunk inwards leaving broad elevated marginal folds. Length 2·7–2·9 mm., width 1·6–1·8 mm. Elongate oval, but narrowing slightly behind, scapulae reduced to broad rounded protuberances, emargination negligible. Surface very strongly convex (without trace of depressions as shown by André & Colas-Belcour), pseudoscutum well defined. Colour brown, smooth, shining. Cervical grooves short, as shallow depressions. Punctations fine, widely dispersed, sparse. Hairs few, fine and very short. Marginal folds broad, punctations as on scutum, with few short hairs (Fig. 1). Ventral plates dark brown, median plate with few short hairs, finely but sparsely punctate. Anal and adanal plates carried round posterior extremity of body; anal plate with parallel sides for the greater part of its length but tending to converge slightly posteriorly; adanal plates almost triangular, slightly longer than anal plate, with lateral margins convex; hairs few and short posteriorly. Pregenital plate pentagonal, rounded in front and extending to front edge of coxa I. Large jugular plates on each side of pregenital plate (Fig. 2).

Legs. Long, with moderate to long spinose hairs. Coxa I subtriangular with slight but distinct indication of a postero-internal spur. All other coxae lacking internal spurs. Small, distinct external saliences on coxae I–III (Fig. 8). Surface of coxae smooth but with moderate-sized punctations. Tarsi I–IV long, tapering with very mild subapical hump on tarsus I (Fig. 6). Length of tarsus I, 0·90 mm., metatarsus I, 0·6 mm. Length of tarsus IV, 0·88 mm., metatarsus IV, 0·67 mm.

Spiracular plate (Fig. 7). Circular, large, diameter 0·38 mm. Large number of goblets of moderate size. Macula either anterior or antero-ventral.

Genital opening. Level with second intercoxal spaces. Covered by a convex apron.

Host associations. Found free in the nests of *Pachyptila desolata* and *Pelecanoides* sp.

Distribution. Port Hopeful (Kerguelen Islands), West Bay (Heard Island), Laurens Peninsula (Heard Island).

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Immunity in trypanosomiasis

IV. Immuno-conglutinin in animals infected with *Trypanosoma brucei*

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The assessment of the effect of chemotherapy in trypanosomiasis depends on accurate diagnosis, which is often difficult to achieve with existing methods. In addition to existing microscopical methods applied to blood, gland juice or cerebrospinal fluid it would be advantageous if a serological method could be developed which could indicate whether during chemotherapy trypanosomes were destroyed or had only temporarily disappeared from the circulation. Studies on immuno-conglutinin in sera of man and various animals carried out by Coombs and his co-workers in Cambridge in the last decade showed that the immuno-conglutinin which is produced in the serum of man and animal following an antigenic stimulation disappears in a short period of time after an antigenic stimulation ceases. Preliminary studies by Ingram, Barber, McLean, Soltys & Coombs (1959) on immuno-conglutinin in bacterial, viral and protozoal infections in laboratory experimental animals showed the most marked response in infection caused by *Trypanosoma brucei*. They also showed that animals maintained the raised level of immuno-conglutinin, not only in the acute stage but also in the chronic stage of infection. In view of these results, experiments were designed to study the presence of immuno-conglutinins in sera of animals infected with *T. brucei* and to find whether chemotherapy affects the level of the immuno-conglutinin in the serum. The results of this experiment are reported in this paper.

MATERIALS AND METHODS

Strains. Trypanosoma brucei shinyanga III was used in these experiments.

The strain has been maintained in mice and rabbits by syringe passage. The strain which was passaged every second day through mice will be referred to as 'M' variant whilst the strain passaged through rabbits every 25–28 days of the infection will be referred to as 'R' variant. A strain of the 'M' variant which was made suramin-fast by frequent passage through mice which were treated with subcurative doses of suramin will be referred to as the 'DF' variant. The biological characteristics of these variants are described by Soltys (1957*a*, 1959).

Diluents. In all cases the trypanosomes were diluted in modified Alsever's solution containing 10% of horse serum (Soltys, 1957*b*).

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Drugs. Suramin which is known under various synonyms (Bayer 205, Antrypol, Germanin) was used as an effective chemotherapeutic drug against *T. brucei*. The dose of 50 mg. was used for both rabbits and cats.

Sera. The sera of rabbits and cats were collected at frequent intervals, both before and after infection, and stored at $-20°$ C. until the whole series for each experiment had been collected.

(1) *Method of examining serum for the presence of immuno-conglutinin*

Sera of infected rabbits and cats were tested for the presence of immuno-conglutinin by a slight modification of the method described by Coombs & Coombs (1953).

(*a*) *Preparation of sera to be tested*

Sera to be tested were inactivated by heating at 56° C. for 30 min. then absorbed with an equal volume of packed sheep red blood cells in order to remove any natural antibodies, agglutinating sheep red cells.

(*b*) *Preparation of alexinated sheep red blood cells*

A suspension of 4% sheep red blood cells was mixed with an equal volume of undiluted inactivated bovine serum and incubated at 37° C. for 15 min. The mixture was then centrifuged, the bovine serum discarded and the red cells were washed with saline and resuspended in saline to a concentration of 4%. To the sensitized sheep red cells, horse complement was added (17·5 ml. saline, 2·5 ml. fresh horse serum, 2·5 ml. heat-inactivated horse serum and 2·5 ml. 4% sensitized sheep red cells), and the mixture incubated at 37° C. for 20 min. Then the mixture was centrifuged, the red cells were washed and resuspended in 25 ml. of saline, to give a 0·4% suspension of alexinated sheep cells.

(*c*) *Technique of immuno-conglutinin titration*

Sera to be examined for the presence of the immuno-conglutinin were absorbed with sheep red blood cells (see A) and a series of doubling dilutions of serum were prepared in volumes of 0·1 ml. Then to each dilution 0·1 ml. of 0·4% alexinated sheep red cells (see B) was added and 0·3 ml. saline to bring the total volume to 0·5 ml. per tube.

After 30 min. incubation at 37° C. the tubes were centrifuged at about 1000 r.p.m. for 1 min., then the sedimented red blood cells were resuspended and according to the clumping of red cells the titre of conglutinin was read. The dilution of the serum showing small clumps without free cells was considered as the end point for the titre of the serum.

(2) *Method of examining neutralizing antibody*

The technique of the neutralization test used in this experiment was similar to that described by Soltys (1957*a*).

EXPERIMENTAL

A. *The production of immuno-conglutinin in animals infected with* Trypanosoma brucei

I. *Rabbits*

(*a*) *Production of immuno-conglutinin in rabbits infected with different numbers of organisms.* Pairs of normal adult rabbits were infected with tenfold serial dilutions of a suspension of 'M' variant *T. brucei* ranging from 2×10^2 to 2×10^5 per ml. All rabbits were inoculated intravenously with 1 ml. of a suspension of organisms. Two rabbits were inoculated with a suspension of 2×10^5 trypanosoma which had previously been killed by heating at 56° C. for 30 min. to determine whether the injection of killed organisms stimulated the production of immuno-conglutinin.

The immuno-conglutinin response of these animals is summarized in Table 1. The rabbits which received 2×10^2 or 2×10^3 viable organisms showed an immuno-conglutinin response which reached peak titres at about 30–35 days after infection.

Table 1. *Immuno-conglutinin response of rabbits infected with various numbers of* Trypanosoma brucei *variant 'M'*

Rabbit no.	No. of organisms injected	Immuno-conglutinin titre: Pre-inoculation	7 days	14 days	20 days	34 days
52	2×10^2	4*	16	16	128	512
53	2×10^2	2	16	32	32	256
54	2×10^3	8	16	16	32	128
55	2×10^3	0	16	16	32	256
56	2×10^4	4	64	512	512	256
57	2×10^4	4	16	—	—	—
38	2×10^5	4	4	64	16	—
39	2×10^5	2	0	256	128	—
36	2×10^5 (killed)	0	0	2	2	—
37	2×10^5 (killed)	0	0	0	0	—

—, Not tested.

* The titres are presented as the reciprocal of the highest dilution showing a positive reaction.

while rabbits injected with 2×10^4 or 2×10^5 organisms reached peak titres by about the 14th day. The two rabbits injected with killed trypanosoma failed to show any significant immuno-conglutinin response. The highest titres obtained in infected rabbits were not governed by the number of infectious organisms injected.

The larger dosages of organisms caused a more rapid rise in the titre of immuno-conglutinin, but the ultimate titre obtained was not dependent on the number of organisms in the challenge. This could be explained if the number of organisms increased *in vivo* to a certain critical level regardless of the actual number injected. The slower response of rabbits inoculated with the smaller number of organisms could be due to the longer period required before the numbers had built up in the host to the optimum which would cause the maximum stimulation to immuno-conglutinin production.

(*b*) *Effect of the 'M' and 'R' variants on the immuno-conglutinin response.* Four rabbits were inoculated intravenously with 5×10^3 'R' variant strain of *T. brucei* (highly virulent), and two others were injected with the same number of 'M' variant strain (less virulent) to find whether the adaptation of the organism effected the immuno-conglutinin response of the rabbit.

The results obtained are summarized in Table 2. In this experiment three rabbits which received the 'R' variant showed a rapid immuno-conglutinin response with peak titres at 10–14 days after infection. The two rabbits injected with the 'M' variant showed a much slower response with peak titres at about 30 days.

Table 2. *Immuno-conglutinin response of rabbits infected with 'R' or 'M' variant of* Trypanosoma brucei

		Immuno-conglutinin titre					
Rabbit no.	Variant	Pre-inoculation	7 days	10 days	14 days	20 days	30 days
34	R	1	256	1024	1024	1024	—
35	R	0	256	512	512	512	—
59	R	2	32	256	512	256	256
60	M	0	16	32	128	256	512
61	M	2	4	16	64	128	256

All animals challenged with 5×10^3 viable organisms.
—, Not tested.

Full immuno-conglutinin production was retarded in rabbits receiving the 'M' variant as compared with those receiving the 'R' variant. It would seem that the adaptation of the organism to growth in rabbits effects the speed of the immuno-conglutinin response in infected rabbits but does not govern the height of the titres obtained. This difference in the speed of the response is probably a reflexion of the virulence of the organism. The rabbit-passaged organism would be expected to start multiplying immediately in the rabbit host and reach maximum numbers within a short period of time, thus causing an early optimal stimulation to maximum immuno-conglutinin production. The mouse-adapted strain, on the other hand, probably would have a 'lag phase' in the rabbit host and thus take a longer time to reach the numbers required for maximum stimulation to immuno-conglutinin production.

II. *Cats*

Two cats were infected by the intravenous injection of 2×10^5 'M' and 'DF' variants of *T. brucei*. Serum samples were obtained at intervals to find if infection of cats with *T. brucei* stimulates immuno-conglutinin production.

The results presented in Table 3 show that a marked immuno-conglutinin response also occurs in cats infected with trypanosomes. The conglutinating activity of the sera rose from 'natural' titres of 4 and 2 to peak titres of 256 within 30 days.

III. *Mice*

Ingram *et al.* (1959) have already shown that mice produce immuno-conglutinin in response to bacterial infections. Experiments were undertaken to find whether a similar response occurred in mice infected with *T. brucei*.

Groups of five mice were injected intraperitoneally with tenfold serial dilutions of the 'M' variant of *T. brucei*. Each mouse was bled 10 days after infection and the individual serum samples titrated for immuno-conglutinin.

Table 3. *Immuno-conglutinin response of cats infected with* Trypanosoma brucei *variants 'M' and 'DF'*

		Immuno-conglutinin titre						
		Pre-inoculation						
Cat name	Variant	1	2	12 days	20 days	27 days	30 days	36 days
Tibby	Drug-resistant	—	4	64	64	128	256	256
Blackie	Drug-sensitive	2	2	64	128	128	256	256

Infected with 2×10^5 organisms.

Table 4. *Immuno-conglutinin response of mice infected with* Trypanosoma brucei *variant 'M'*

	No. of injected organisms					
	5×10^0	5×10^1	5×10^2	5×10^3	5×10^4	5×10^5
Immuno-conglutinin titre	0	0	0	2·5	*	*
	0	0	0	2·5	40	*
	0	0	0	10	80	*
	0	2·5	0	10	160	*
	2·5	2·5	10	20	160	*

* Animal died.

The immuno-conglutinin titres of five groups of mice are presented in Table 4. The animals inoculated with a small number of organisms did not show an abnormal distribution of titres, but as the number of injected trypanosomes increased, the immuno-conglutinin titres rose markedly.

These data show that mice do respond to infection with *T. brucei* by the production of immuno-conglutinin and that the more severe the infection, the more marked is the immuno-conglutinin response.

B. *Immuno-conglutinin in infected animals after treatment with suramin*

I. *Immuno-conglutinin levels in animals infected with* Trypanosoma brucei *and treated with suramin*

The pairs of rabbits infected with various numbers of *T. brucei* were utilized for this experiment to test the effect of drug therapy on the immuno-conglutinin titre. About 28–35 days after infection, one animal from each pair of rabbits was

treated with a therapeutic dose of suramin. Serum samples were collected at intervals after treatment from both the treated and untreated rabbits.

Table 5 presents the pertinent data from this experiment. The conglutinating activity in the serum of treated rabbits decreased rapidly after therapy, and within 1–3 weeks had returned to the 'normal' or pre-infection level of the animal, while in non-treated rabbits a high titre of immuno-conglutinin was retained.

Table 5. *Immuno-conglutinin levels in rabbits infected with 'M' variant of* Trypanosoma brucei *and treated with suramin*

		Immuno-conglutinin titre						
			Time after infection in days		Time after treatment in days			
Animal no.	Therapy	Pre-infection	28 (6)*	33 (1)	3	7	10	20
52	Treated	4	256	128	4	4	4	4
53	Not treated	2	256	128	64	64	64	64
54	Treated	8	128	128	16	8	8	8
55	Not treated	0	128	128	64	64	64	64

* Number in parentheses is the number of days before treatment.

II. *Immuno-conglutinin levels in rabbits infected with drug-resistant* Trypanosoma brucei *and treated with suramin*

Two rabbits and one cat were infected with a drug-resistant 'DF' variant of *T. brucei* and, when the immuno-conglutinin response had reached its peak, were treated with suramin. Serum samples were obtained at intervals after treatment to find whether the immuno-conglutinin titre dropped in these 'unsuccessfully treated' animals.

The results of the immuno-conglutinin titrations are presented in Table 6. The immuno-conglutinin level in three rabbits infected with the drug-sensitive strain showed a marked drop after therapy, while the immuno-conglutinin in the two rabbits infected with the drug-resistant (DF) strain remained at high levels of activity.

The cat ('Tibby') infected with the 'DF' variant retained a high titre of immuno-conglutinin, but the cat infected with the drug-sensitive strain showed a decline of only one dilution. The cat ('Tibby') showed no improvement and died of trypanosomiasis after 73 days, while the other cat showed a clinical improvement after treatment, but it became ill after 4 months and died of trypanosoma infection 3 weeks later. The treatment of this animal had been unsuccessful and it was probably the continuous presence of trypanosomes in the animal which caused the immuno-conglutinin titre to remain at a high level.

Drug therapy of rabbits infected with a drug-sensitive strain of *T. brucei* resulted in a subsequent decline in immuno-conglutinin titres and the conglutinating activity of the serum usually returned to the low 'natural' level within 7–20 days after treatment. Drug therapy of the cat infected with a drug-sensitive variant failed to cause a significant reduction of immuno-conglutinin titre, although a

clinical improvement was noted in this animal. However, the failure of the treatment of this animal became evident when it subsequently died of trypanosoma infection. On the other hand, drug therapy of rabbits and one cat ('Tibby') infected with a drug-resistant strain failed to reduce the high immuno-conglutinin titres in the sera of these animals.

Table 6. *Immuno-conglutinin levels in animals infected with drug-sensitive or drug-resistant strains of* Trypanosoma brucei *and treated with suramin*

		Immuno-conglutinin titre						
			Time after infection in days		Time after treatment in days			
Animal	Infecting organism	Pre-infection	22 (6)*	27 (1)	3	7	10	20
56 (rabbit)	Drug-sensitive	4	256	256	64	16	4	4
60 (rabbit)	Drug-sensitive	0	128	256	16	8	8	4
61 (rabbit)	Drug-sensitive	2	256	512	32	16	8	4
Blackie (cat)	Drug-sensitive	2	128	256	256	—	128	—
62 (rabbit)	Drug-resistant	8	128	256	—	128	128	—
63 (rabbit)	Drug-resistant	16	256	256	—	256	256	—
Tibby (cat)	Drug-resistant	4	128	256	256	—	256	—

—, Not tested.
* Number in parentheses is number of days before treatment.

C. *Comparative studies with immuno-conglutinin and neutralizing antibody*

I. *Time of production of immuno-conglutinin and neutralizing antibody in rabbits infected with variant 'M' or 'R'*

Studies were carried out on the correlation between the titre of immuno-conglutinin and neutralizing antibody in the sera at various intervals of time after infection of rabbits with two variants of *T. brucei*. The results of this experiment are summarized in Table 7.

The results shown in Table 7 indicate that immuno-conglutinin appears in the serum much sooner than neutralizing antibodies. It has also been shown that immuno-conglutinin appears much sooner in rabbits infected with the more virulent 'R' variant, than in rabbits infected with 'M' variant, which is less virulent for rabbits. No difference, or a very slight difference, was observed in the time of production of neutralizing antibody in animals infected with the two strains.

II. *The levels of immuno-conglutinin and neutralizing antibody in animals infected with drug-sensitive and drug-resistant variants after treatment with suramin*

Animals infected with the two variants of *T. brucei* were treated with 50 mg. of suramin at the peak of infection when the titres of both immune substances were high. Then the sera of the animals were tested for the persistence of both neutralizing and conglutinating activities at various intervals of time. The results of this experiment are summarized in Table 8.

The results clearly demonstrated the marked drop in the titre of immuno-conglutinin in animals infected with a drug-sensitive variant but only a slight drop in the titre of neutralizing antibody. On the other hand, all animals infected with the drug-resistant variant showed no significant change in the level of both antibodies 10 days after treatment. It appears from this experiment that the test

Table 7. *Immuno-conglutinin and neutralizing antibody responses of rabbits infected with 'R' or 'M' variant of* Trypanosoma brucei

Animal	Variant	Test	Time after infection: 7 days	10 days	14 days	20 days	30 days
34	R	Immuno-conglutinin	256	1024	1024	1024	—
		Neut. antibody	5	10	80	320	640
35	R	Immuno-conglutinin	256	512	512	512	—
		Neut. antibody	2	5	40	160	640
59	R	Immuno-conglutinin	32	256	512	256	256
		Neut. antibody	2	10	80	160	320
60	M	Immuno-conglutinin	16	32	128	256	512
		Neut. antibody	0	10	40	80	320
61	M	Immuno-conglutinin	4	16	64	128	256
		Neut. antibody	2	5	80	160	640
54	M	Immuno-conglutinin	4	16	16	32	128
		Neut. antibody	5	10	80	160	640

—, Not tested.

Table 8. *Immuno-conglutinin and neutralizing antibody levels in animals infected with drug-sensitive or drug-resistant strains of* Trypanosoma brucei *and treated with suramin*

Animal	Infecting organism	Test	Before* treatment	After† treatment
56	Drug-sensitive	Immuno-conglutinin	256	4
		Neut. antibody	640	80
60	Drug-sensitive	Immuno-conglutinin	256	8
		Neut. antibody	320	160
61	Drug-sensitive	Immuno-conglutinin	512	16
		Neut. antibody	640	80
Blackie	Drug-sensitive	Immuno-conglutinin	256	128
		Neut. antibody	160	40
62	Drug-resistant	Immuno-conglutinin	256	256
		Neut. antibody	160	160
63	Drug-resistant	Immuno-conglutinin	256	128
		Neut. antibody	640	160
Tibby	Drug-resistant	Immuno-conglutinin	256	256
		Neut. antibody	640	640

* Sample obtained 27 days after infection, 1 day before treatment.
† Sample obtained 10 days after treatment.

for the level of immuno-conglutinin in treated animals may give an indication of the success of treatment—provided that the level in the sera before treatment is also known.

Further studies in the field are of course necessary to confirm the results obtained in experimental animals.

CONCLUSIONS AND SUMMARY

Studies on immuno-conglutinin levels in animals infected with *T. brucei* have shown that immuno-conglutinin appears in rabbits infected with *T. brucei* 'M' variant within 7 days after infection and reaches its peak titre within 30 days. In rabbits infected with the more virulent 'R' variant immuno-conglutinin levels rose more rapidly and reached peak titres within 10–14 days after infection.

High levels of immuno-conglutinin are detectable in the sera of rabbits and cats infected with trypanosomes before a significant amount of neutralizing antibody is produced. This difference is most marked in animals infected with the antibody-resistant variant 'R' which is more virulent for rabbits.

In animals treated effectively the immuno-conglutinin level dropped to its pre-infection level, while the titre of neutralizing antibody only decreased to a slightly lower level.

No change in the titres of immuno-conglutinin and neutralizing antibody was observed after treatment of animals infected with a drug-resistant strain.

These results are very promising for further field trials and introduce a new approach and method for testing the efficiency of chemotherapy in trypanosomiasis.

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The effect of changes in the osmotic pressure upon *Hammerschmidtiella diesingi* (Hammerschmidt, 1838) with reference to the survival of the nematode during moulting of the cockroach

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It was observed that *Leidynema appendiculata* (Leidy, 1850) and *Hammerschmidtiella diesingi* (Hammerschmidt, 1838; Chitwood, 1932), which are nematodes parasitic in the hind-gut of *Blatta orientalis* L., could survive in the hind-gut when the cockroach moulted and it was decided to see what effect moulting of the cockroach had upon the nematodes.

In moulting cockroaches there is a certain amount of drying in the gut which presumably increases the osmotic pressure of the gut contents. The nematodes in moulting cockroaches are found inside the cast peritrophic membrane of the gut, have a shrunken appearance and are rather inactive. By examining the nematodes at various times after the insects had moulted it was found that the cast peritrophic membrane becomes broken up in the middle section of the hind-gut. The nematodes, which have at this stage become more active, escape from the broken membrane and move up to their normal position in the hind-gut.

The shrunken appearance of the nematodes suggested that they were in a hypertonic environment. It was therefore decided to observe the effect of various concentrations of 1·0 M sodium chloride, 1·0 M sucrose, and sea water upon the adult female nematodes to determine if they were capable of surviving long exposures to these solutions and to see if there was subsequent recovery in isotonic solutions.

METHOD

Adult females of *Hammerschmidtiella diesingi* were used in the following experiments because they are not so active as *Leidynema appendiculata* and were therefore easier to measure.

The nematodes were taken from adult *Blatta orientalis* and immersed in the experimental solutions in solid watch-glasses. The watch-glasses were filled to the brim with the solution, covered with a glass plate and incubated at 24° C. The edges were sealed with petroleum jelly to prevent evaporation and so keep the osmotic pressure of the solution constant during the experiment. The nematodes were observed under a dissecting microscope and measurements made of their length, without removing them from the watch-glasses. Measurements were made by twisting thread along the length of a camera lucida image of the nematode

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to determine the increase or decrease in length (Stephenson, 1942). Because of their undulating movements and small size, this was the only method which could be used to determine the effect of the experimental solutions on the nematodes. Each experiment was carried out upon five individuals and a mean was taken. The solutions used were various dilutions of 1·0 M sodium chloride, 1·0 M sucrose, and sea water.

Dead nematodes were easily noticed as they very quickly showed signs of decay.

RESULTS

The effect of immersion of adult females of *Hammerschmidtiella diesingi* in various dilutions of 1·0 M sodium chloride, 1·0 M sucrose and sea water, for certain periods of time, are shown in Figs. 1–3. It was found that the rate and extent of

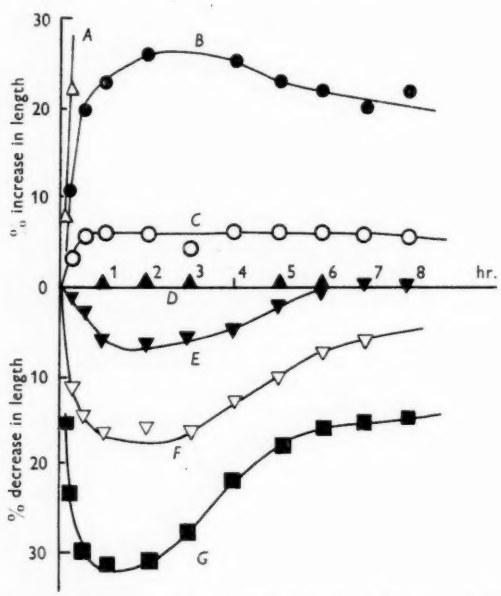

Fig. 1. The percentage increase or decrease in length of females of *Hammerschmidtiella diesingi* in distilled water and in various molar concentrations of sodium chloride (NaCl). *A* = distilled water; *B* = 0·05 M-NaCl; *C* = 0·08 M-NaCl; *D* = 0·15 M-NaCl; *E* = 0·2 M-NaCl; *F* = 0·3 M-NaCl; *G* = 0·5 M-NaCl.

increase or decrease in length varied with the strength of the solution employed. After 2–3 hr. in hypertonic sodium chloride solutions there was a reduction in the amount of shrinkage, the recovery being more rapid, at first, in the more concentrated solutions (Fig. 1). After 6 hr. in 0·2 M sodium chloride the nematodes had regained their normal length (Fig. 1), whereas it took nematodes in 0·3 M sodium chloride up to 24 hr. to regain their original length and even longer for those in 0·5 M sodium chloride. The nematodes in 0·2 and 0·3 M sodium chloride were still alive after 24 hr. but most of those in 0·5 M were dead. In 0·05 and 0·08 M sodium

chloride there was some recovery towards the normal length (Fig. 1) but the nematodes had not regained their original length within 24 hr., although they were still alive. The smallest changes in length occurred in 0·15 M sodium chloride which must be approximately isotonic (Fig. 1). Similarly, nematodes placed in hypertonic solutions of sea water recovered their normal length after a period of shrinkage (Fig. 3), whereas there was little recovery in hypotonic solutions. It

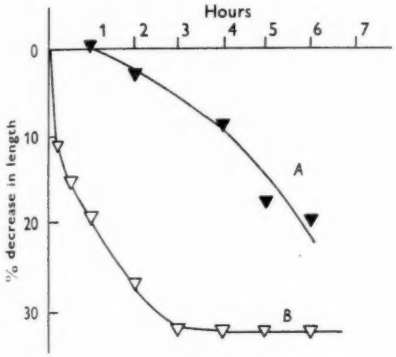

Fig. 2

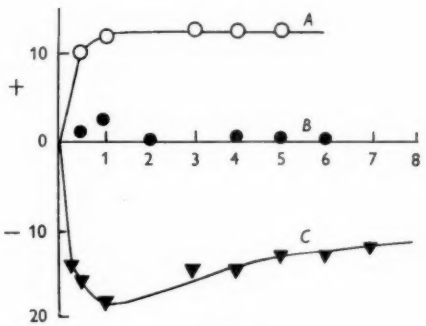

Fig. 3

Fig. 2. The percentage decrease in length of females of *Hammerschmidtiella diesingi* in 0·4 M (*A*) and 0·6 M (*B*) sucrose.

Fig. 3. The percentage increase (+) or decrease (−) in length of females of *Hammerschmidtiella diesingi* in various dilutions of sea water. *A* = 12 %; *B* = 30 %; *C* = 50 % sea water.

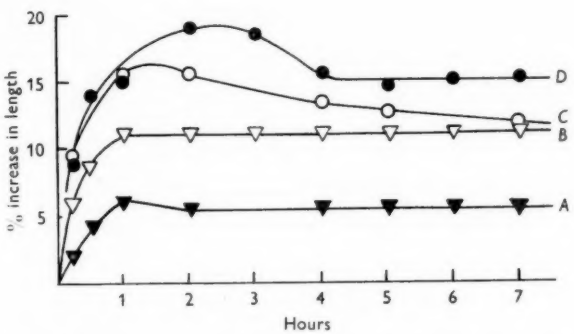

Fig. 4. Showing the percentage increase in length, in 0·15 M sodium chloride, of females of *Hammerschmidtiella diesingi* after 24 hr. in 0·2 M (*A*); 0·3 M (*B*); and 0·4 M (*C*) NaCl; and of females of *H. diesingi* taken from moulting cockroaches (*D*).

was found that 30 % sea water was isotonic (Fig. 3). However, nematodes placed in hypertonic sucrose solutions (Fig. 2) continued to shrink and no reduction in the amount of shrinkage occurred within 24 hr.

Nematodes which had regained their normal length after 24 hr., or less, in 0·2 and 0·3 M sodium chloride, increased in length, as if they were in a hypotonic

solution, when placed in normally isotonic sodium chloride (0·15 M) (Fig. 4) or 30% sea water. Similarly, it was found that females of *H. diesingi*, taken from the hind-gut of a moulting cockroach and placed in 0·15 M sodium chloride, increased in length as if they were in a hypotonic solution (Fig. 4). This increase in length of the nematodes taken from moulting cockroaches and placed in 0·15 M sodium chloride is approximately the same as in those nematodes taken from 0·4 M sodium chloride, after they had regained their normal length, and placed in 0·15 M sodium chloride (Fig. 4).

DISCUSSION

It has been shown by Hobson, Stephenson & Beadle (1952) and Hobson, Stephenson & Eden (1952) that the osmotic pressure, conductivity and chloride concentration of the body fluid of *Ascaris lumbricoides* were almost in direct proportion to those of the external medium used in their experiments (20–40% sea water) and that this nematode is nearly poikilosmotic under these conditions. Mueller (1929) has demonstrated that certain non-electrolytes can pass through the body wall of *Ascaris*.

The experiments of Pannikar & Sproston (1941) with *Angusticaecum* sp., an ascarid from a turtle, indicated a permeability to sodium chloride. Although the nematode lost and gained salts in relation to the salts of the medium, it could remain hypertonic to tap water.

Stephenson (1942) showed that water can pass both into and out of the body of *Rhabditis terrestris*, under the influence of osmotic forces, with a resulting increase or decrease in the size of the body. He also found that after prolonged immersion of *R. terrestris* in distilled water, the swelling of the body was reduced and there was a return to normal movement. He suggested that this recovery was due to the existence of an active method of osmotic regulation as recovery was inhibited by injury or when cyanide was present. *H. diesingi* rapidly expands and ruptures when placed in distilled water; however, in 12% sea water, 0·05 and 0·08 M sodium chloride there is no marked decrease in the initial swelling after 24 hr. although the nematodes are still alive. This seems to suggest that the active method of osmotic regulation found in *R. terrestris* is either absent or works much more slowly in *H. diesingi*. Stephenson also found that when *R. terrestris* was immersed in hypertonic solutions there was an increase in the internal osmotic pressure, largely due to the removal of water from the body, and that after a considerable time, about 20 hr., there was a reduction in the amount of shrinkage. A similar phenomenon has been observed in *H. diesingi* immersed in hypertonic sea water and sodium chloride solutions, there being complete recovery in length after 6 hr. in 0·2 M sodium chloride, and after 24 hr. in 0·3 M sodium chloride and in 50% sea water. When nematodes which have recovered their original length in a hypertonic solution are placed in 30% sea water or 0·15 M sodium chloride, both of which are normally isotonic, there is an increase in length as if the worms were in a hypotonic solution. This seems to suggest that the nematodes immersed in hypertonic sodium chloride solutions have, after a period of shrinkage due to water loss, taken up ions from the surrounding medium.

thereby increasing the osmotic pressure of the body fluid, and so taken up water from the medium with a resulting decrease in the amount of shrinkage. These nematodes were unable to recover their normal length within 24 hr. when transferred to a normally isotonic solution which suggests that the excretion of ions from the body fluid does not take place as readily as the uptake of ions. It has been shown that nematodes taken from moulting cockroaches increase in length in 0·15 M sodium chloride, indicating that they have taken up ions from the contents of the hind-gut. There must eventually be a return to the normal length in isotonic media otherwise the nematodes would become more distended after each moult of the cockroach. The increase in length, in 0·15 M sodium chloride, of nematodes taken from moulting cockroaches is approximately the same as the increase in length of nematodes taken from 0·4 M sodium chloride, after they had regained their original length, and placed in 0·15 M sodium chloride (Fig. 4). This does not necessarily signify that the hind-gut contents of moulting cockroaches have an osmotic pressure equivalent to 0·4 M sodium chloride because a certain amount of shrinkage may be caused by other factors.

The variations in length of the nematode inside the moulting cockroach, where changes in the osmotic pressure presumably occur gradually, will not be as great as in the experiments described above where the worms were suddenly transferred from one solution to another of quite different osmotic pressure.

These experiments show that females of *H. diesingi* can survive changes in the osmotic pressure of the external medium and this will help them to survive any osmotic changes which may occur in the hind-gut of the moulting cockroach.

SUMMARY

Females of *Hammerschmidtiella diesingi* can survive in the hind-gut of moulting *Blatta orientalis*. After a period of shrinkage they recover their normal length within 24 hr. in 0·2 and 0·3 M sodium chloride and in 50 % sea water, but not in hypotonic media or in hypertonic sucrose. Females of *H. diesingi*, which have regained their original length in hypertonic media, swell when placed in normally isotonic media; a similar phenomenon occurs with nematodes taken from moulting cockroaches.

I wish to thank Dr P. Tate for his advice and encouragement during this investigation.

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The distribution of glycogen and fat in *Thelastoma bulhõesi* (Magalhães, 1900), a nematode parasitic in cockroaches

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INTRODUCTION

The distribution of glycogen and fat in well-fed and starved nematodes has been the subject of intense investigation by many workers, using quantitative chemical, and semi-quantitative histochemical, methods. Von Kemnitz (1912), Toryu (1933) and Hirsch & Bretschneider (1937) have studied the distribution of glycogen in adults of *Ascaris lumbricoides* and *A. equorum*, using histochemical methods. They found that most of the glycogen is concentrated in the non-contractile parts of the body-wall musculature and in the female reproductive system, although they also found it in the epidermis, including the dorsal, ventral and lateral chords, the intestinal epithelium and the oesophagus. Von Brand (1938) found large amounts of glycogen in the intestinal cells and in the non-contractile part of the muscle cells of the larva of *Eustrongylides ignatus*, parasitic in the tissues of a fish. Enigk (1938) described glycogen distribution in the intestinal epithelium of *Graphidium strigosum*.

Giovannola (1936) found little or no glycogen in the free-living larval stages of certain parasitic nematodes, the reserves being large amounts of fat stored in the wall of the intestine. Rogers (1939) also found large amounts of fat in the free-living larval stages of certain nematodes. According to Flury (1912) the fat in *Ascaris lumbricoides* is mainly to be found in the body wall and the reproductive organs, whereas von Kemnitz (1912) found it chiefly in the epidermis, especially in the chords, and very little in the reproductive organs. Fauré-Fremiet (1913) found fat in the intestinal epithelium, spermatogonia, oogonia and young oocytes of *A. equorum*; Mueller (1929) found it mostly in the epidermis and body-wall musculature in both *A. lumbricoides* and *A. equorum*; but in *Ancylostoma caninum* it is almost entirely confined to the rays of the bursa (Giovannola, 1936).

The nematode chosen for the following investigation was *Thelastoma bulhõesi* (Magalhães, 1900; Travassos, 1929) (Thelastomatidae) which is parasitic in the hind-gut of *Periplaneta americana* L. Certain observations were also made upon the distribution of glycogen and fat in *Hammerschmidtiella diesingi* (Hammerschmidt, 1838; Chitwood, 1932) (Thelastomatidae), which is also parasitic in the hind-gut of *Periplaneta americana*.

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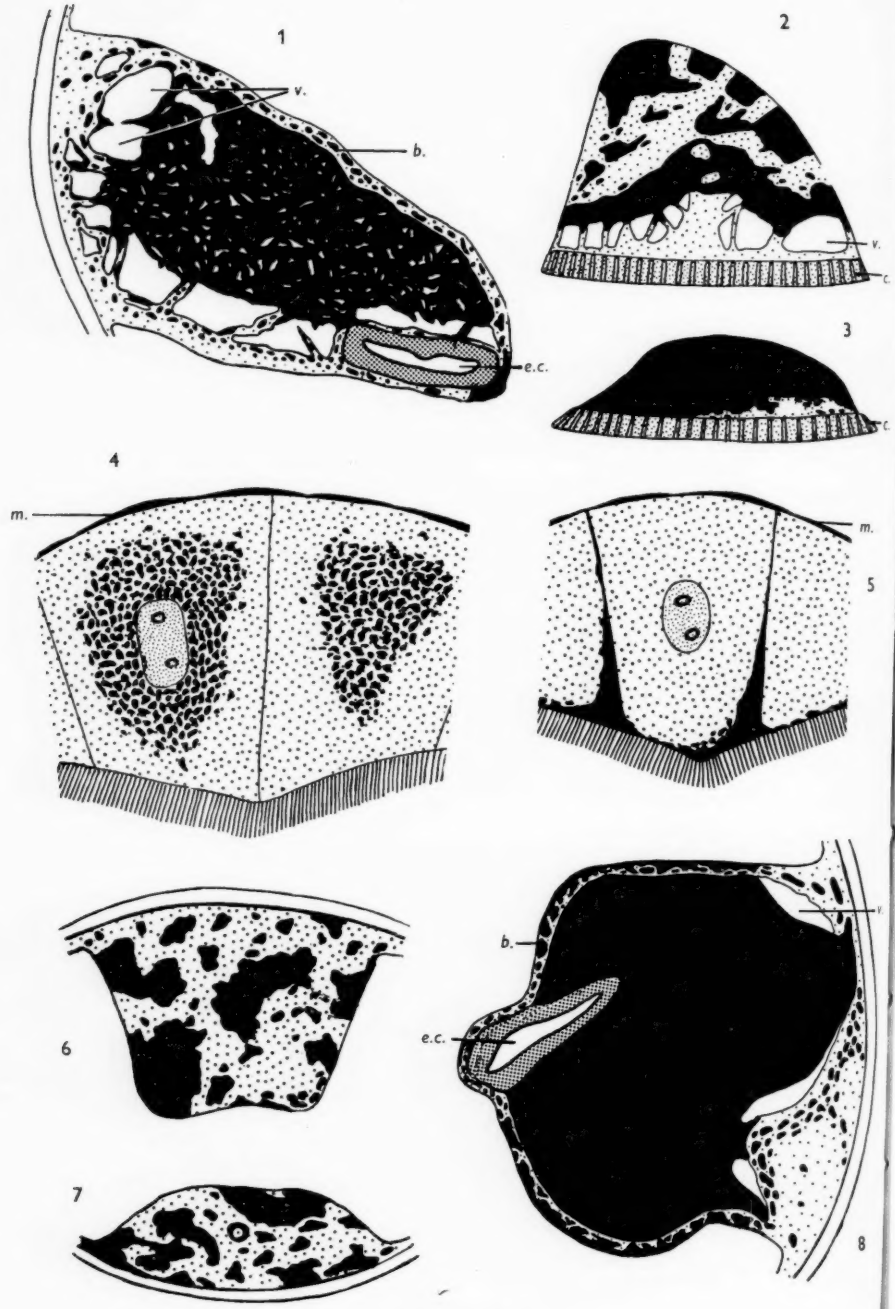

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DISTRIBUTION OF GLYCOGEN

Method. The nematodes were killed and fixed in Carnoy's fixative or Lison's 'Gendre fluid' (Pearse, 1953), at $-10°$ to $-20°$ C. for 24 hr., washed in 70 % alcohol to remove the fixative, and embedded in paraffin wax using the butyl alcohol method given by Goodey (1957). Sections were cut at 5μ and floated on 90 % alcohol on the albuminized slide. These sections were stained with Best's carmine stain. Every tenth section was removed from the ribbon and mounted on a separate slide to act as a control. These control sections were treated with saliva for 1 hr. before staining to remove any glycogen present.

Results

Cuticle and hypodermis

The cuticle is glycogen-free. Glycogen is present in small amounts in the hypodermis, mainly in the region of the muscle cells posterior to the valvular bulb.

Dorsal and ventral lines

Glycogen is present in large amounts in these chords in the form of large patches and coarse granules (Figs. 6, 7).

Lateral lines

The lateral lines are one of the most important storage areas of glycogen in this nematode. The excretory canal is always free from glycogen, but the remainder of the lateral line is densely filled with it, although the anterior regions contain slightly less than the rest of the chord. The main storage area, which stains as a solid red mass in sections, is enclosed by the boundary layer of the lateral line (Figs. 1, 8). This boundary layer contains large amounts of glycogen in the main body of the animal (Fig. 8), but not so much in the anterior end (Fig. 1). The mass of glycogen is usually separated from the hypodermis by vacuoles which are probably areas of fat storage. The lateral lines in the region of the anus are enlarged and full of glycogen.

Figs. 1–8. Transverse sections of *Thelastoma bulhõesi* stained with Best's carmine to show distribution of glycogen. *b.*, Boundary layer of lateral line; *c.*, contractile border; *e.c.*, excretory canal; *m.*, muscle net covering intestine; *v.*, vacuoles (? sites of fat storage). The solid black areas represent glycogen.

Fig. 1. Lateral line anterior to valvular bulb.

Fig. 2. Muscle cell anterior to valvular bulb.

Fig. 3. Muscle cell posterior to valvular bulb.

Fig. 4. Intestinal cells of central mid-intestine.

Fig. 5. Intestinal cells of anterior mid-intestine.

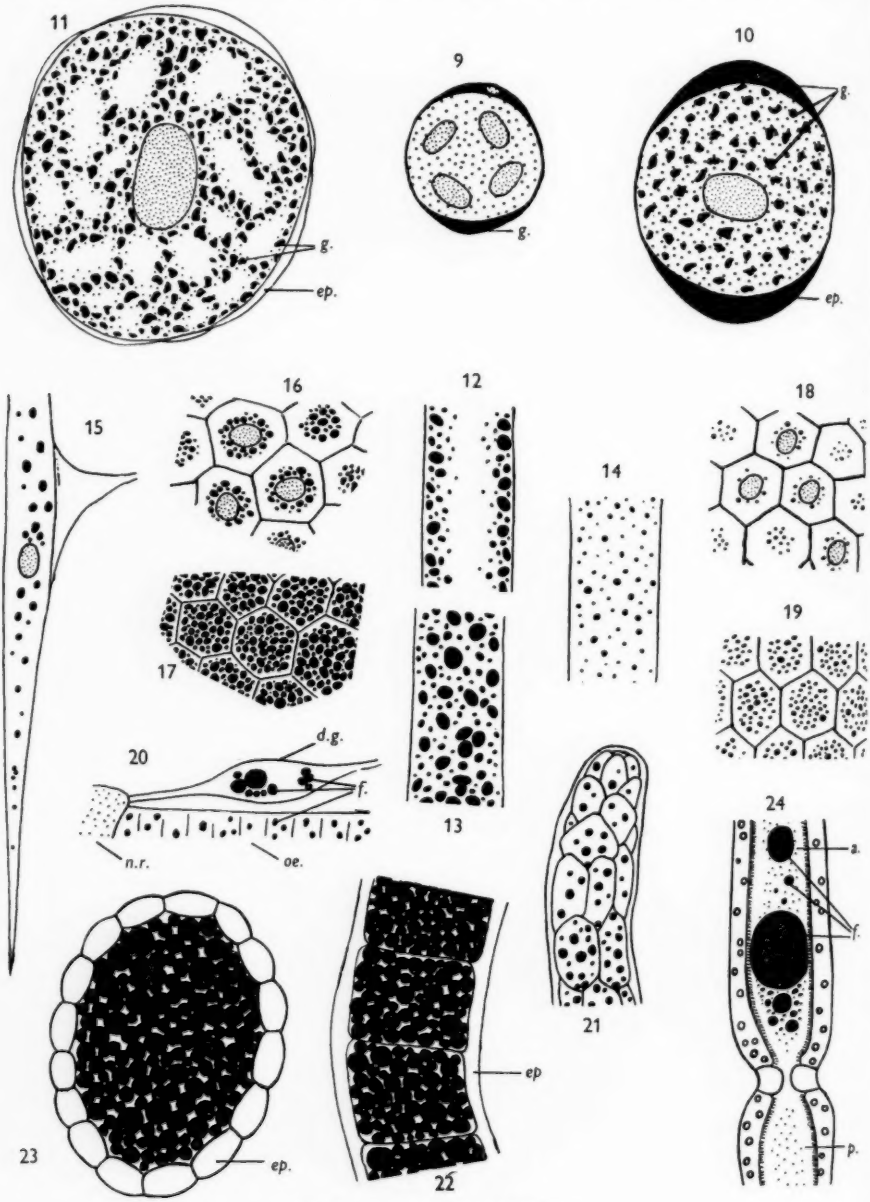
Fig. 6. Dorsal line in region of valvular bulb.

Fig. 7. Ventral line in region of valvular bulb.

Fig. 8. Lateral line in region of valvular bulb.

Body-wall muscle cells

These contain large amounts of glycogen, the non-contractile part of the muscle cell being densely filled with it. Glycogen was not demonstrated in the contractile border. The muscle cells in the anterior region of the nematode usually contain



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less glycogen than the more posterior muscles (Table 1; Figs. 2, 3). Small vacuoles, which are probably areas of fat storage, lie between the contractile and the non-contractile parts of the muscle cell.

Oesophagus

In the oesophagus glycogen is found in the muscles and increases in amount from the mouth towards the isthmus, there being very little near the anterior end. In the valvular bulb glycogen is found in the muscles which operate the valve.

Intestine

The anterior end of the intestine is swollen to form a cardia, the cells of which are free from glycogen. Most of the glycogen in the intestine is found in the cells of the middle intestine, with a decrease in the amount present towards the cardia and the posterior intestine. In the first part of the middle intestine, after the cardia, the glycogen tends to accumulate against the walls of the cell adjoining the lumen and the next cell (Fig. 5). In the middle part of the middle intestine the glycogen is found as a compact mass of small lumps encircling the nucleus and away from the cell walls; the amount of glycogen in this area is usually large (Table 1; Fig. 4). The posterior intestine contains only small amounts of glycogen scattered in the cells (Table 1).

The muscle net which covers the intestine is rich in glycogen, as are the depressor and dilator muscles of the anus.

Figs. 9–11. Transverse sections of *Thelastoma bulhõesi* stained with Best's carmine to show distribution of glycogen (black).

Fig. 9. Germinal zone of ovary showing glycogen in epithelium only.

Fig. 10. Growth of zone ovary showing glycogen in both epithelium and oogonia.

Fig. 11. Oogonia in ovary prior to fertilization, glycogen absent from epithelium.

Figs 12–23. *Thelastoma bulhõesi* stained with oil red O to show distribution of fat (black). Figs 12–22 whole mount preparations. Fig. 23 Transverse section.

Fig. 12. Lateral line.

Fig. 13. Dorsal line.

Fig. 14. Dorsal line after 12 days starvation.

Fig. 15. Body wall muscle in region of nerve ring.

Fig. 16. Surface view of cells of cardia of intestine.

Fig. 17. Surface view of cells of mid-intestine.

Fig. 18. Surface view of cells of cardia after 12 days starvation.

Fig. 19. Surface view of cells of mid-intestine after 12 days starvation.

Fig. 20. Dorsal ganglion, lateral view.

Fig. 21. Germinal zone of ovary.

Fig. 22. Growth zone of ovary.

Fig. 23. Growth zone of ovary.

Fig. 24. Male of *Hammerschmidtiella diesingi*. Intestine containing fat globules (black) in the lumen.

a., Anterior intestine; *d.g.*, dorsal ganglion; *ep.*, epithelium; *f.*, fat globules; *g.*, glycogen; *n.r.*, nerve ring; *oe.*, oesophagus; *p.*, posterior intestine.

Gonads

Female. The germinal zone of the ovary contains little, if any, glycogen, but the germinal zone epithelium is well supplied with it (Fig. 9). The amount of glycogen in the epithelium becomes progressively smaller along the ovary into the growth zone until eventually there is no trace of it; whilst the amount of glycogen in the oogonia increases along the length of the ovary (Figs. 9–11). In the oogonia the glycogen is found as small coarse lumps (Fig. 11). The ova and oogonia never become very rich in glycogen.

The oviduct appears to be glycogen-free but small amounts are found surrounding the nucleus of the uterine cells. The ovijector is well supplied with glycogen.

Male. The epithelium of the testis is full of glycogen but the germinal zone, the growth zone and the spermatozoa are apparently glycogen-free. Small amounts are found in the glandular walls of the seminal vesicle and in the upper regions of the vas deferens.

The nerve ring with associated ganglia, the coelomocytes and the rectal glands are all glycogen-free.

Table 1. *The distribution of glycogen in* Thelastoma bulhõesi (*female*) *during starvation*

	Period of starvation			
	0 days	6 days	9 days	12 days
Body muscles				
Ant.	+ + +	+ + +	+ + +	+ + +
Post.	+ + + +	+ + + +	+ + + +	+ + + +
Dorsal line	+ +	+ +	+	0
Ventral line	+ +	+ +	+	0
Lateral line	+ + + +	+ + +	+ +	+
Oesophagus	+ +	+	+	+
Valvular bulb	+ +	+	+	+
Intestine				
Cardia	0	0	0	0
Middle	+ + +	+ + +	+ +	+
Posterior	+	+	0	0

Ant. = distribution anterior to the valvular bulb. Post. = distribution posterior to the valvular bulb. + to + + + + = amount of glycogen. 0 = no glycogen.

DISTRIBUTION OF FATS

Method. The distribution of fats has been determined by staining frozen sections of formalin-fixed material, embedded in gelatin, with sudan III and IV, or oil red O, in 70% alcohol; and by staining whole mounts using a modification of Goodey's (1930) method. The nematodes were killed, fixed and stained with a hot saturated solution of oil red O in 70% alcohol. After 5–10 min. in this solution the nematodes were transferred to a watch-glass containing distilled water

with a drop of glycerine mixed in it. The water was allowed to evaporate over a period of 2–3 days so that the nematodes were eventually left in glycerine only. They were then mounted in pure glycerine. The oil red O has been found to show up fat droplets, especially the finer droplets, much more clearly than the sudan dyes.

Results

Cuticle and hypodermis

Fat was not detected in the cuticle. In the hypodermis small amounts are found scattered generally throughout the animal. Larger amounts are found along the dorso-lateral and ventro-lateral lines and in the tail.

Dorsal and ventral lines

These structures contain large amounts of fat along the whole length of the nematode (Table 2; Fig. 13) and are one of the largest areas of fat storage.

Table 2. *The distribution of fat in* Thelastoma bulhõesi (*female*) *during starvation*

	Period of starvation			
	0 days	6 days	9 days	12 days
Body muscles	+ +	+	+	0 to +
Dorsal line				
Ant.	+ + + +	+ + + +	+ + +	+ + +
Post.	+ + + +	+ + +	+ +	+
Ventral line				
Ant.	+ + + +	+ + + +	+ + +	+ + +
Post.	+ + + +	+ + +	+ +	+
Lateral line				
Ant.	+ + + +	+ + + +	+ + +	+ + +
Post.	+ + + +	+ + +	+	0 to +
Oesophagus	+ +	+	0	0
Intestine				
Cardia	+ + +	+ +	+ − 0	0
Middle	+ + + +	+ + +	+ +	+
Posterior	+ + +	+ +	+	0

Ant. = distribution anterior to the valvular bulb. Post. = distribution posterior to the valvular bulb. + to + + + + = amount of fat. 0 = no fat.

Lateral lines

The lateral lines also contain large amounts of fat (Table 2) but the droplets are situated along the edges of the lateral lines, leaving a clear area between the two zones of fat (Fig. 12), and in the boundary layer. These droplets do not extend into the swollen glycogen filled area of the lateral chord.

Body-wall muscle cells

The contractile border of the muscle cells is apparently free from fat. The non-contractile part of the cell contains small amounts which lie close to the contractile border (Table 2; Fig. 15).

Oesophagus

There are small amounts of fat lying between the muscles (Fig. 20) in the oesophagus but very little, if any, in the valvular bulb. No fat has been detected in the oesophagus of the male or of the female fourth-stage larva.

Intestine

Fat droplets are found in large quantities in the intestinal cells. There are large amounts in the cells of the cardia (Table 2; Fig. 16), and in the posterior intestine (Table 2), but even larger amounts are found in the cells of the middle intestine (Table 2; Fig. 17). The fat droplets congregate around the nucleus leaving the walls of the cells free (Fig. 16). The intestine of the male and of the female fourth-stage larva contains relatively much more fat than the intestine of the adult female. Large globules of fat are sometimes found in the lumen of the intestine of the female, male and fourth-stage larva, and especially in the male of *H. diesingi* (Fig. 24). When sudan black B in 70 % alcohol was added to 30 % sea water in which was mounted one living male of *H. diesingi*, fat present in the lumen of the intestine was regurgitated through the valvular bulb and out of the mouth where it rapidly took up the stain.

Gonads

Female. The germinal zone epithelium, growth zone epithelium, oviduct and uterus are free from fat. The germinal zone of the ovary contains very little fat (Fig. 21) but farther down the ovary the amount rapidly increases, the growth zone and the developing oogonia being extremely rich in fat (Figs. 22, 23). The ova also contain large amounts of fat. Small amounts are occasionally found in the ovijector.

Male. The male reproductive system appears to be compietely free from fat.

Nerve ring and associated ganglia

The nerve ring and most of the associated ganglia are free from fat; but the dorsal ganglion contains large fat droplets (Fig. 20) and certain cells in the lateral ganglia also contain small amounts of fat.

Rectal glands and coelomocytes

The rectal glands contain small amounts of fat. Very occasionally small amounts of fat have been observed in the coelomocytes.

THE EFFECT OF STARVATION ON THE DISTRIBUTION OF FOOD RESERVES

It was decided to try to obtain a table of the distribution and amount of glycogen and fat present in females of *Thelastoma bulhõesi* starved for known periods of time in 30 % sea water at 30° C. and in females taken directly from the cockroach. The object of this table was to try to devise a standard with which the nematodes,

in various culture media, could be examined to see if they were utilizing the food in the media or whether they were living upon their food reserves.

Method. Females of *T. bulhõesi* were incubated at 30° C. in sealed watch glasses containing 30 % sea water. The distribution of glycogen and fat was determined as described earlier.

It was found that the amount of glycogen varied in well-fed nematodes and so the results in Tables 1 and 2 were compiled, for each column, from the amounts present in five nematodes.

Results

The distribution of glycogen in well-fed females of *T. bulhõesi*, taken straight from the cockroach (0 days), and in nematodes starved for 6, 9, and 12 days is shown in Table 1. The distribution of fat, in well-fed and starved nematodes, is shown in Table 2 and in Figs. 12–23.

There is a steady loss of both glycogen and fat in starving females of *T. bulhõesi*. Fat is removed most rapidly from the intestine and from the dorsal, ventral and lateral lines posterior to the region of the valvular bulb, whilst glycogen is removed most rapidly from the lateral lines and the intestine. The glycogen and fat in the gonads appears to remain unchanged.

Estimating the amount of fat present in whole-mount preparations was found to be the most convenient method of determining whether the food reserves of the nematode were being used.

DISCUSSION

The distribution of glycogen and fat in *T. bulhõesi* has been found to be essentially the same as in *Ascaris lumbricoides* and *A. equorum*, but the amounts of these reserve food materials in certain tissues and organs are rather different.

In the muscle cells of the body wall the chief form of stored food is glycogen which almost completely fills the non-contractile part of the cell. The amount of glycogen present in the muscle cells of the anterior end is less than is found in the more posterior muscle cells. Von Kemnitz (1912), who found a similar phenomenon in *Ascaris*, suggested that this may be because of the greater activity of these muscles in performing the searching movements of the mouth region, in which case they would be constantly using up their food reserves. Small amounts of fat are found between the glycogen mass of the non-contractile part of the cell and the contractile border, although none was observed surrounding the nucleus as observed in *Ascaris* by von Kemnitz (1912) and Mueller (1929). Neither glycogen nor fat is found in the contractile border of the muscle cell which is in agreement with the findings of von Kemnitz (1912) and Toryu (1933).

Although the dorsal and ventral lines contain large amounts of glycogen their chief form of food reserve is fat. The lateral lines, however, are one of the largest storage areas for glycogen in *Thelastoma*, the swollen parts of the chords being densely filled with it, as in *Ascaris* (von Kemnitz, 1912). The walls of the excretory canal are completely free from fat and glycogen. Fat in the lateral lines is found between the main body of the chord and the hypodermis and in the boundary

layer of the chord. The amount of fat is similar to that found in the dorsal and ventral chords; but the distribution differs in that the fat tends to be confined to the edges of the lateral lines (Fig. 12). Von Kemnitz (1912) found the chords and the general hypodermis to be one of the chief storage areas for fat in *Ascaris*.

The intestine of *Thelastoma* is an important storage organ for reserve food. In the adult female the cells of the middle intestine are rich in both glycogen and fat. The cardia, or anterior intestine, although rich in fat is glycogen-free for most of its length. In the posterior intestine and rectum the amount of glycogen present in the cells decreases rapidly away from the middle intestine; however, although there is a decrease in the number and size of the fat droplets, the amount of fat present is still quite large. Chitwood & Chitwood (1950) identified fat as the chief form of stored food in the intestine of *Cephalobellus papilliger* (Thelastomatidae) and *Blatticola blattae* (Thelastomatidae) but did not mention the amount of glycogen present. In *Ascaris lumbricoides* and *A. equorum* von Kemnitz (1912) and Hirsch & Bretschneider (1937) found glycogen in large quantities in the intestine but only small amounts of fat. Toryu (1933), however, found only small amounts of glycogen in the intestine of *A. equorum*. In the male and the female fourth-stage larva of *Thelastoma* the cells of the intestine are extremely rich in fat. Small globules of fat are sometimes found in the lumen of nematodes, a phenomenon observed by Giovannola (1936) in the free-living larval stages of certain parasitic nematodes. Globules of fat have been found occasionally in the lumen of the female and male of *T. bulhõesi* and quite frequently in the lumen of the male of *H. diesingi*, especially after starvation for 24 hr. The globules in the lumen of the male of *H. diesingi* are often large and can be observed moving back and forth along the lumen as a result of movements of the intestinal wall (Lee, 1958*a*) and of the body as a whole. The disgorging of fat through the mouth of a male of *H. diesingi* is interesting because it shows that under certain adverse conditions the contents of the intestine can pass through the valvular bulb and be carried forwards along the length of the oesophagus to the exterior.

One can put forward various suggestions as to why these globules of fat are found in the lumen. It is possible that the fat droplets in the cells of the intestine have been shed into the lumen to be broken down by the lipase of the gut (Lee, 1958*b*) into easily absorbed and transported food; the fat droplets may have been shed from the cells into the lumen to be passed to the exterior; or they may be the less easily digested remains of fatty material drawn into the lumen from the hind-gut contents of the host. Of these suggestions I am inclined to regard the first as the most probable as these fat globules often appear in the lumen after 24 hr. starvation.

In the female reproductive organs of *T. bulhõesi* the distribution of glycogen and fat is essentially the same as in *Ascaris*. The amounts of glycogen in the ovaries, oogonia and ova of *Thelastoma* are, however, much smaller than those found in *Ascaris* by von Kemnitz (1912) and Toryu (1933). The epithelial cells of the germinal zone of the ovaries, which are extremely rich in glycogen, appear to supply glycogen to the rapidly growing cells of the ovary which, in the germinal zone, are glycogen-free. There are noticeable amounts of glycogen in the oogonia

of the growth zone, whilst there is a decrease in the amount present in the epithelial cells of this zone. However, although fat is absent from the epithelial cells and scarcely present in the germinal zone, it is the most abundant food reserve in the ovaries. Von Kemnitz (1912) was unable to find fat in the ovaries of *Ascaris* but Flury (1912) and Fairbairn (1955*a*) have shown that the ovaries are extremely rich in fat.

The epithelial cells of the testis probably supply glycogen to the developing spermatocytes, which are glycogen-free, while the glycogen present in the walls of the seminal vesicle and vas deferens is probably passed to the spermatozoa. No fat was observed in any part of the male reproductive tract.

The presence of fat in the nerve cells of nematodes was observed by von Kemnitz (1912), and confirmed by Mueller (1929) who found large drops of fat in certain cells in the lateral ganglion of *Ascaris* and small droplets in almost all of the nerve cells. Fat droplets have been observed in a pair of cells in the lateral ganglion of *T. bulhõesi* and also in the dorsal ganglion where the amount of fat present is quite large.

Giovannola (1936) and Rogers (1939) found that fat in the free-living infective stages of certain parasitic nematodes was utilized whilst they were waiting to gain entry into a new host. Mueller (1929) maintained small pieces of the lateral line of *Ascaris* in hanging drops of the body fluid for up to 8 days and found that not only the glycogen but also the fat had disappeared from the sections although the tissue was still apparently healthy. Hirsch & Bretschneider (1937) found a decline in the fat content of the intestinal cells of *Ascaris* during starvation. Enigk (1938) showed the presence of both glycogen and fat in the cells of the mid-gut of larvae of *Graphidium strigosum*, but found there was no apparent change in the fat content of the fasting larva although the glycogen became greatly reduced. Enigk suggested that the fat is a useless by-product of the metabolism. Von Brand (1941) reported little utilization of fat reserves by starving adults of *A. lumbricoides*.

Hobson (1948), after reviewing the results obtained by various workers, stated that fat present in parasitic nematodes is a true reserve food which is not normally used as a source of energy by the nematode because of the low oxygen tension usually present in the intestine, and because large amounts of glycogen are more readily available. He also stated that if sufficient oxygen is available, at certain stages of the life history, the fat is almost certainly broken down. Fairbairn (1955*b*) found that *Ascaris* eggs are extremely rich in fats and that this provides a concentrated source of nourishment for the developing embryo and for some time after the infective stage is reached. He also found that these fats include a considerable amount of the lower volatile acids which closely resemble those which the adult female excretes as waste fermentation products, and he states that some of these are perhaps incorporated into the ovarian triglycerides instead of being excreted and are subsequently used as sources of energy during the oxidative metabolism of the developing embryo.

It has been definitely shown, in the above experiments on females of *T. bulhõesi*, that fat present in the nematode, except in the reproductive organs, is utilized

at the same time as glycogen during starvation. It should be remembered that Rogers (1941) has shown that *Strongylus edentatus* and *A. lumbricoides* both possess a lipase in extracts of the intestine and that Lee (1958*b*) has shown that *Leidynema appendiculata* (Thelastomatidae) also possesses a lipase in the intestine and it is therefore reasonable to assume that since there is a mechanism for breaking down fats in these nematodes then the breakdown products are utilized in some way.

SUMMARY

1. The distribution of glycogen and fat in *Thelastoma bulhõesi* has been shown to be essentially the same as in *Ascaris lumbricoides*.

2. The chief storage areas of glycogen are the muscle cells and the lateral lines, with smaller amounts in the dorsal and ventral lines, the intestine and the oesophagus.

3. The chief storage areas of fat are the dorsal, ventral and lateral lines, the intestine, the oocytes, oogonia and ova.

4. Fat has been found in the lumen of the intestine of several nematodes, especially in the males of *Hammerschmidtiella diesingi* after 24 hr. starvation.

5. Both glycogen and fat in females of *T. bulhõesi* were considerably reduced, except in the gonads, after 12 days starvation.

6. Staining whole mounts with oil red O was found to be the most convenient method of determining if the food reserves were being used or not.

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The growth of *Cooperia curticei* (Giles, 1892), a nematode parasite of sheep

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The growth of nematodes is characterized by a succession of four moults, at each of which the old cuticle is detached and often shed, to be replaced by a new cuticle. Each moult may be preceded by a lethargus, during which there is no increase in length, although differentiation and reorganization of tissue may occur. The growth of nematodes is therefore a discontinuous process and the shape of the growth curve will to a large extent depend upon the moults and the number and duration of the periods of lethargus. Amongst nematodes which may be cited as examples are *Haemonchus contortus* (Veglia, 1915), *Ancylostoma caninum* (McCoy, 1930) and *Nippostrongylus muris* (Twohy, 1956).

The object of this study was to examine the growth of a parasitic nematode and to determine the influence of the lethargus and moult upon the shape of the growth curve. The species selected, *Cooperia curticei*, was prominent in ecological studies on nematodes parasitic in sheep (Tetley, 1949), and it was as a supplement to this work that information on the growth curve was required.

MATERIALS AND METHODS

The experiments were carried out with spring-born Romney-cross lambs about 9 months old. These animals were born during a period of 3 weeks and were reared indoors under conditions which excluded accidental nematode infection, except for occasional *Strongyloides papillosus* and *Nematodirus* sp. After weaning, the sheep were fed on lucerne hay, supplemented with a proprietary concentrate feed until 10 days prior to infection.

Infective 'larvae' of *C. curticei* were cultured from sheep dung stored in glass jars for 9 days at 37° C. Initially, 15 sheep each received 5000 larvae, and on the same day a further 14 sheep each received 11,000 larvae (Table 1). Subsequently, a further 8 sheep were infected on the one day, half of which received 10,000 larvae each and the remainder 70,000 larvae each (Table 1). Less than 30 min. were taken to infect the sheep on both occasions, and on each occasion the infective larvae were collected from faecal cultures which had been prepared on the same day. After infection, sheep were selected at random for slaughter (Table 1). As soon as each sheep was killed, the contents of the small intestine were expressed into separate jars and preserved immediately by addition of cold formalin to a

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concentration of about 10%. Subsequently, worms were collected from this material and preserved in cold 5% formalin and glycerine.

The life history and morphology of *C. curticei* have been described and the various stages are readily recognized (Andrews, 1939). The mature adult is approximately seven times longer than the infective larva and, since length is readily measured, it was selected as the index of growth. Specimens were first mounted in 5% formalin on a slide under a coverslip, the image of each worm was projected on to a sheet of paper and the length was recorded by drawing a line from end to end down the central axis. The lines were measured with a map-measurer and the length of each worm calculated. The reliability of this method was determined by ten successive measurements of nine adult worms 4–8 mm. long and nine fourth-stage larvae 1·6–2·3 mm. long. The standard deviation per observation for drawing was 0·045 and 0·047 mm. respectively, and for measuring, 0·028 and 0·01 mm. respectively.

Measurements were made of approximately 200 male and 200 female worms selected at random from each population. When identification of sex could not be made with certainty, 200 larvae were measured.

Table 1. *Synopsis of the experiment, showing the numbers of larvae received by individual sheep and the age of the infections when the hosts were killed*

Age of infection when host was killed (days)	Ear tag numbers of infected sheep			
	Received 5000 larvae	Received 10,000 larvae	Received 11,000 larvae	Received 70,000 larvae
2	32	—	47	—
3	44	—	39	—
4	16	—	14	—
5	4	—	35	—
6	37	40	24	28
7	—	10	41, 43	23
8	8	42	19	34
9	2	25	7	49
10	48	—	—	—
11	36	—	38	—
12	26	—	—	—
14	52	—	51	—
16	33	—	30	—
18	9	—	29	—
20	—	—	6	—
22	18, 27	—	—	—

RESULTS

More than 15,000 worms were measured, the mean lengths for each population and sex plotted and a growth curve drawn by hand (Fig. 1).

Growth of third-stage larvae

Forty-eight hours after infection, third-stage larvae were recovered from the small intestines of sheep 32 and 47. These larvae had undergone the second ecdysis and the mean length of both populations was 0·72 mm. This was shorter than the

infective larvae, which had a mean length of 0·76 mm. and a standard deviation of 0·03 mm.

After a further 24 hr. in the host, i.e. 72 hr. after infection, more than three-quarters of both populations (sheep 39 and 44) had passed the third ecdysis and reached the fourth stage. Many of those larvae which were still in the third stage were longer than the third-stage larvae recovered 24 hr. earlier. For example, the mean length of third-stage larvae in sheep 39 was $0{\cdot}97 \pm 0{\cdot}14$ mm., and in sheep 44, $0{\cdot}75 \pm 0{\cdot}16$ mm. In both sheep many larvae were longer than 1·0 mm., although still in the third stage. On the other hand, some fourth-stage larvae were less than 0·9 mm. long, and a few were as short as 0·65 mm.

Third-stage larvae were found neither in sheep 14 and 16, killed 96 hr. after infection, nor in older infections.

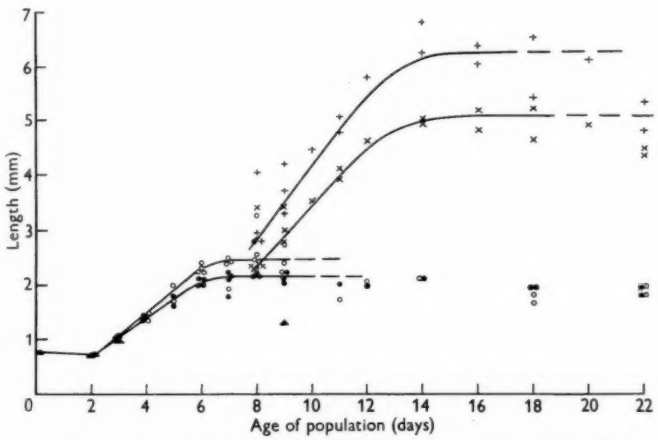

Fig. 1. Growth curve of *C. curticei* in sheep. Curves were fitted by hand to points which represent mean lengths of worms measured. 'Sexes not differentiated' includes only third-stage larvae at 0 and 2 days and only fourth-stage larvae at 9 days. At 3 days more than 75 % of larvae were fourth stage and the remainder third stage. +, Adult females; ×, adult males; ○, fourth-stage females; ●, fourth-stage males; ▲, sexes not differentiated.

Growth of fourth-stage larvae

Four days from the inception of parasitism, males and females are distinguishable by the shape of the tail (Andrews, 1939) and separate growth curves were therefore drawn (Fig. 1). Females were commonly longer than males from the same population and this difference became greater as the worms grew. At the end of the fourth stage the mean length of females exceeded that of males by about 0·3 mm.

Adult worms were first recovered 8 days after infection, and the minimum duration of the fourth stage was therefore about 5 days. The maximum duration could not be determined, because small numbers of larvae in the late fourth state were found in most infections which had been established more than 9 days. These

larvae commonly represented between 1% and 8% of the total population, although in one sheep, no. 18, killed 22 days after infection, about 15% of the worms were still in the fourth stage (Fig. 2).

The majority of the larvae which were measured on the seventh and eighth days after infection were approximately the same length as those recovered at 6 days and the growth curve therefore formed a plateau, which suggests that the larvae were in a lethargus at this time. However, a few 'giant' fourth-stage larvae, which occurred in the four sheep killed 8 days after infection, were between 0·5 and 2·0 mm. longer than 6- or 7-day-old larvae.

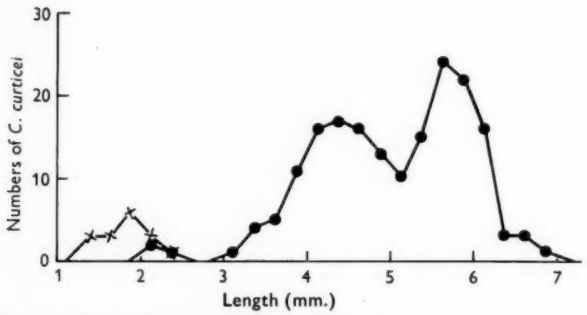

Fig. 2. The distribution of length of female *C. curticei* recovered from a 22-day-old infection. Sheep no. 18. ×—×, fourth stage; ●—●, fifth stage.

Growth in the fifth stage

Fifth-stage or adult worms were first recovered from the sheep which were killed 8 days after infection, in which they formed about a third of the population. At 9 days, the majority of worms had passed the fourth ecdysis and entered the fifth stage and continued to grow until about 14 days after infection.

Uterine eggs were first seen in about 4% of females from sheep 38, killed 11 days after infection. In sheep 26, killed 12 days after infection, one-third of the females contained eggs and the mean number of eggs per female was 8. Fourteen days after infection, the mean number of eggs per female was 17 (sheep 51) and 21 (sheep 52) and at 16 days, 27 (sheep 52).

However, in certain of the oldest infections (sheep 9, 18, 27 and 29) some females were devoid of eggs, and their mean length was always less than the mean length of worms which contained eggs. In one instance, in which about half of the females were without eggs, the frequency distribution of length for the whole population was bimodal (Fig. 2). Whenever females without eggs were present, a similar proportion of males in the same population possessed colourless spicules and the mean length of these worms was always less than the mean length of those with dark spicules.

Relationship between numbers of infective larvae and growth of the nematodes

One of the four sheep which received 70,000 infective larvae (Table 1), was killed 9 days after infection. The worms were markedly undeveloped and accurate identification of sex was not possible (Fig. 1). Similarly, the mean lengths of male and female worms in sheep 23, killed 7 days after infection with 70,000 larvae, were shorter than the mean lengths of populations of the same age derived from fewer infective larvae. However, there was no consistency in this relationship, for the populations in the two remaining animals which were infected with 70,000 larvae were either the same length (sheep 28), or longer (sheep 34), than similar aged worms derived from fewer larvae.

DISCUSSION

A succession of growth, lethargus, ecdysis and growth has been shown to occur in *Haemonchus contortus* (Veglia, 1915) and in the first and second stages of *Ancylostoma caninum* (McCoy, 1930): other instances may be cited. On the other hand, there are species which depart considerably from this pattern. In *Meloidogyne* spp. for example, growth is suspended while three successive moults take place (Bird, 1959) and growth of *Ascaridia galli* in the chicken appears to be a continuous process uninterrupted by a lethargus (Ackert, 1931). However, in *Cooperia curticei* the growth of the parasitic stages was essentially a succession of growth, ecdysis and growth. Details of the life history agreed with the data given by Andrews (1939), except that the third ecdysis was 24 hr. earlier than he reported.

The lethargus which preceded the fourth ecdysis commenced about 6 days after infection and, in the majority of larvae, terminated 8 days after infection, forming a plateau in the growth curve (Fig. 1). However, there was a similar plateau in this curve for the first 2 days after infection, during which larvae not only failed to grow but actually decreased in length by approximately 25μ. Although this decrease can be attributed to the loss of the second-stage sheath, the possibility that the larvae themselves decreased in length cannot be excluded, for it occurs in *Haemonchus contortus* (Stoll, 1940).

Failure of third-stage larvae to grow during the initial 48 hr. in the host seems unlikely to be a consequence of an unfavourable environment, for the rate at which other species of larvae migrate from the sheep's mouth to the small intestine (Mönnig, 1926; Whitlock, personal communication) suggests that most third-stage larvae of *Cooperia curticei* would be in the small intestine for at least 75 % of this time. A similar lag in the initiation of growth has been observed in infections with cestodes of the genus *Diphyllobothrium* and it has been suggested that in this instance it represents a period of physiological adaptation (Archer & Hopkins, 1958).

The time occupied by the fourth lethargus, together with the lag in the initiation of growth, totalled approximately 96 hr. Growth of the majority of adult worms ceased about 14 days after infection, so that for about one-third or one-quarter of this time there was no increase in length.

Some of the fourth-stage larvae recovered 3 days after infection were as short

as 0·65 mm., which indicated that larvae were able to pass the third ecdysis with little or no increase in length. Similarly, the larvae of *Cooperia curticei* which Andrews (1939) studied 'appeared to increase very little in length in the parasitic part of the third stage'. On the other hand, a majority of third-stage larvae had increased in length and many were longer than 1·0 mm. Unlike growth in the fourth and fifth stages, growth in the third stage was variable and was not an essential preliminary to the third ecdysis.

Although a majority of fourth-stage larvae moulted and entered the fifth stage about 8 days after infection (Fig. 1), many failed to do this and were found as late fourth-stage larvae in a number of infections, including the oldest examined (Fig. 2). It seems unlikely to be fortuitous that the majority of these larvae were in the late fourth stage. Retarded development of other species has often been reported to precede or follow an ecdysis, e.g. retarded larvae of *Haemonchus placei* and *Ostertagia circumcincta* are unsuccessful in passing beyond the early fourth stage (Bremner, 1956; Sommerville, 1954), while under certain conditions larvae of *Trichostrongylus retortaeformis* in rabbits are arrested in the late third stage (Michel, 1952). It seems possible that worms which are close to an ecdysis are more likely to be adversely affected by unfavourable features of the environment than they are at other times.

Retarded development has frequently been observed when the host has been subjected to successive infections from the same species (Michel, 1952). However, the sheep used in this experiment had either no previous experience of infection by nematodes or were only infected with isolated *Strongyloides papillosus*, *Nematodirus* sp., or both. Retarded larvae of *Ostertagia circumcincta* and *Haemonchus placei* have also been found in hosts which were devoid of parasites prior to experimental infection (Sommerville,1954; Bremner, 1956). In none of these instances was it known whether retarded development was a consequence of an environment which was initially unsuitable, or whether the infection itself rendered the environment unfavourable.

SUMMARY

A growth curve of the parasitic stage of *Cooperia curticei* was drawn from measurements of worms collected from sheep between 2 and 22 days after infection. Exsheathed third-stage larvae did not grow during the first 48 hr. in the host. However, growth commenced between 48 and 72 hr. after infection, immediately prior to the third ecdysis. Following this ecdysis, the young fourth-stage larvae increased in length until the sixth day after infection, when growth ceased with the onset of the fourth lethargus. In most worms, this lethargus was terminated by the fourth ecdysis 8 days after infection. However, even in the oldest infections, late fourth-stage larvae were found which had been unable to enter the fifth stage. The first worms to reach the fifth stage ceased growth between 12 and 14 days after infection.

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A new paruterinid cestode, *Lallum magniparuterina* gen. et sp.nov. from the intestine of a common teal, *Nettion crecca* Linn.*

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(*Received* 11 *June* 1959)

Three complete specimens of a paruterinid cestode were recovered from the intestine of a common teal, *Nettion crecca* Linn., shot in the environs of district Bareilly, U.P.

The worms are small, being 31–42 mm long, with a maximum width of 1·39 mm. The scolex is large and slightly wider than long; it is 0·87–0·90 mm. long and 1·05–1·30 mm. wide. The rostellum and rostellar hooks are absent. The suckers

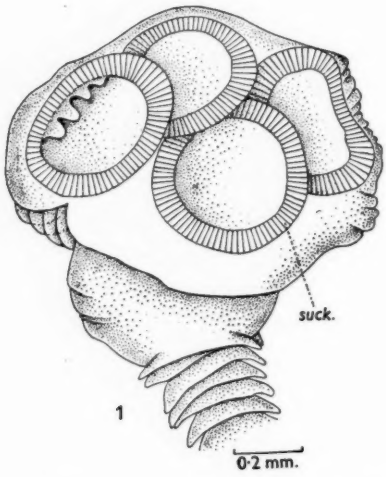
Table 1. *Main characters of the unarmed genera belonging to the subfamily* Paruterinae *Fuhrmann*, 1907

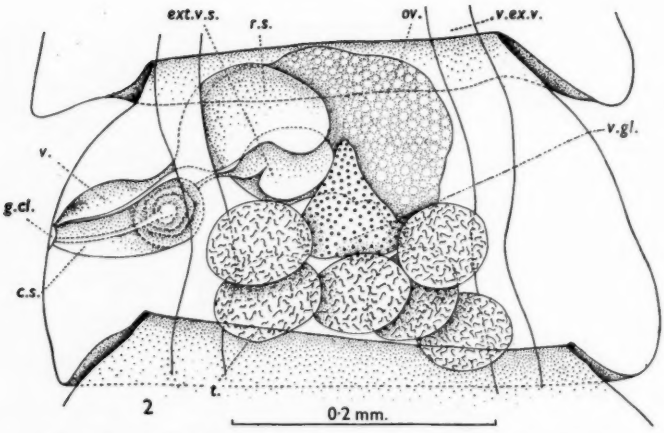
	Genital pore	Genital ducts in relation to excretory vessels	Testes	Vagina in relation to cirrus sac	Uterus	Paruterine organ
Anonchotaenia Cohn, 1900	Alternating irregularly	Ventral or between	Few (4–16)	Posterior or ventral	Small, oval or round with few eggs	Finger-like lateral to uterus
Anomaloporus Voge & Davis, 1953	Alternating irregularly	—	Few (6–14)	Posterior	Transversely elongated sac later with pouches	Large circular anterior to uterus
Metroliasthes Ransom, 1900	Alternating irregularly	Between	Numerous (20–40)	Posterior	Two fused sacs	Conical fibrous anterior to uterus
Octopetalum Baylis, 1914	Alternating irregularly	—	Numerous	Posterior	—	Anterior to uterus
Rhabdometra Cholodkovsky, 1906	Alternating irregularly	Between	Numerous	Posterior	Saccular	Anterior to uterus
Lallum n.g.	Unilateral	Ventral	Few (5–7)	Anterior	Large with many eggs	Posterior to uterus

are highly muscular, almost spherical and prominently displayed in the anterior region of the scolex. They are 0·47–0·60 mm. in diameter. The neck is insignificant, but in one of the specimens it is quite prominent and is 0·13 mm. long. The lateral appendages are present on all the segments, but are more prominent on the mature

* Part of thesis approved by the University of Lucknow for the award of the Ph.D. degree.

and gravid segments than on the immature ones. The mature segments are 0·19–0·23 mm. long and 0·45–0·49 mm. wide; the gravid segments are 0·42–0·48 mm. long and 0·50–0·55 mm. wide. The genital pores are unilateral and are located near the middle of the lateral margin of the segments. A small genital cloaca is present. The genital ducts pass ventral to the poral longitudinal excretory vessels.



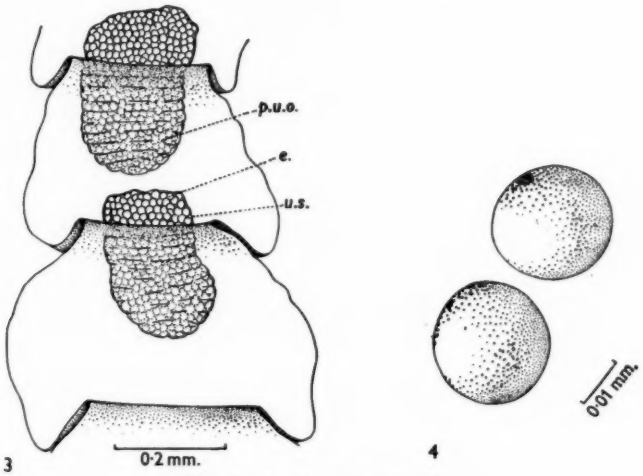


Text-fig. 1. Scolex of *Lallum magniparuterina* gen. et sp.nov.
Text-fig. 2. Mature segment.
For key to abbreviations see p. 272.

The cirrus sac is elongated and extends as far as the ventral poral longitudinal excretory vessel and in some cases even crosses it. It measures 0·10–0·13 mm. in length and 0·032–0·041 mm. in width. The external vesicula seminalis is well developed; it measures 0·05 mm. in length and 0·075 mm. in width and almost touches the ovary and the vitelline gland. Within the proximal globular

portion of the cirrus sac the cirrus is like a watch spring with only a few coils. There are five to seven testes, which occupy the posterior half of the segment behind the ovary; they lie between the ventral longitudinal excretory vessels and measure 0·15–0·18 mm. in diameter.

The ovary is well developed and without lobes; it lies in the anterior half of the segment and is stretched transversely between the ventral longitudinal excretory vessels; in some segments it extends a little into the posterior portion of the preceding segment. It measures 0·18–0·20 mm. across its widest part. The vagina is located anterior to the cirrus sac and is continued into a large receptaculum seminis which measures 0·073–0·075 mm. in length and 0·10–0·13 mm in width. The vitelline gland is more or less triangular in shape and measures 0·093 mm. in length and 0·083 mm. in maximum width; a portion of it lies dorsal to the ovary. The uterus initially develops in the semigravid segments as an irregular, transversely located structure in the anterior half of the segment within the longitudinal



Text-fig. 3. Gravid segments.
Text-fig. 4. Egg.

excretory vessels. A paruterine organ develops from the hinder median border of the uterus as an irregular, slightly lobed structure extending as far as the posterior border of the segment. In the fully gravid region of the strobila, where the segments become more elongated, the paruterine organ develops further and extends into the anterior half of the succeeding segment in the form of a large sac. The eggs are spherical in shape and measure 0·026 mm. in diameter. The embryonic hooks were not observed.

From the characters given above, it is clear that the present form belongs to the subfamily Paruterinae Fuhrmann, 1907, of the family Dilepididae Fuhrmann, 1907. The absence of a rostellum and rostellar hooks in the present form easily separates it from all the genera in the subfamily, except *Anonchotaenia* Cohn, 1900, *Anomaloporus* Voge & Davis, 1953, *Metroliasthes* Ransom, 1900, *Octopetalum*

Baylis, 1914, and *Rhabdometra* Cholodkovsky, 1906, all of which are also unarmed. The present form can, however, be distinguished from all the genera just mentioned by its possession of unilateral genital pores, a vagina anterior to the cirrus sac and a paruterine organ attached posterior to the uterus and extending into the following segment, a character which is unique and is not shared by any other genera of the subfamily so far known. The present form can be further separated from *Anonchotaenia*, *Anomaloporus* and *Metroliasthes* by the form of the uterus, which is, in the form here described, large and saccular with numerous eggs, whereas in *Anonchotaenia* it is small and oval or round with few eggs, in *Anomaloporus* it is a transversely elongated sac which later develops uterine pouches and in *Metroliasthes* it has the form of two fused sacs. Further *Anonchotaenia* possess nematode-like (vermiform) embryos and in *Metroliasthes* the genital ducts pass between the poral longitudinal excretory vessels and in this respect differ from those of the present form.

SUMMARY

A description is given of a new species of paruterinid Cestode, belonging to the subfamily Paruterinae Fuhrmann, 1907, of the family Dilepididae Fuhrmann, 1907, to which the name *Lallum magniparuterina* gen. et sp.nov. is given. It is parasitic in the intestine of the common teal, *Nettion crecca* Linn.

The author wishes to express his gratitude to Professor M. B. Lal, under whom the present investigations were carried out, and to the authorities of the University of Lucknow for certain facilities.

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KEY TO ABBREVIATIONS

c.s., cirrus sac; *e.*, egg; *ext.v.s.*, external vesicula seminalis; *g.cl.*, genital cloaca; *ov.*, ovary; *p.u.o.*, paruterine organ; *r.s.*, receptaculum seminis; *suck.*, sucker; *t.*, testis; *u.s.*, uterine sac; *v.*, vagina; *v.ex.v.*, ventral longitudinal excretory vessel; *v.gl.*, vitelline gland.

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A simple apparatus for the artificial feeding of *Rhodnius prolixus* (Hemiptera, Reduviidae)

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I. INTRODUCTION

During a study on the nutritional requirements of the blood-sucking bug, *Rhodnius prolixus*, it became necessary to construct a feeding apparatus so that a large variety of liquids could be fed across a membrane to all stages of the insect. The unit was reduced to its simplest terms. Special efforts were made to avoid the use of animal membranes with their attendant disadvantages and this was easily accomplished by the use of thin rubber.

A number of observations made on the behaviour of *Rhodnius* during artificial feeding are described in this paper.

II. MATERIALS AND METHODS

The apparatus consists of a small thistle funnel—about 15 ml. capacity—covered at the mouth by a membrane, and sealed at the narrow end with a cork stopper. The funnel is supported in the neck of a small bottle in a 400 ml. beaker filled with water kept at a temperature of about 40° C. The water level is regulated so that the mouth of the funnel with its dry membrane protrudes through the surface. Insects brought into contact with the membrane commenced to feed immediately.

The feeding unit does not contain a thermostatic device such as that in the elaborate feeding apparatus described by Nicolle (1941) for the feeding of the related reduviid bug, *Triatoma infestans*, and temperature is maintained by means of a small burner.

(*a*) *Membranes*

Feeding through animal membranes such as mouse skin, and rat and rabbit caecae was accomplished successfully, but such membranes have obvious disadvantages associated with their preparation and preservation. They also have to be constantly renewed. Instead of these, membranes of thin Durex rubber, used originally by Nicolle (1941), proved to be admirable in all respects. Rubber membranes are easily procurable and of good stability. Moreover, they can be used for weeks on end.

Before the commencement of artificial feeding, the membrane was washed in distilled water and dried between sheets of filter-paper. It was then placed over

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the mouth of the thistle funnel and secured by rubber bands wound tightly round the neck. The fitting of the membrane was done in such a way as to allow a certain amount of distension of the membrane by the enclosed liquid. Feeding was more successful through a slightly slack membrane than through one tightly stretched.

(*b*) *Procedure*

After the fitting of the membrane, the funnel was filled with the dietary liquid by a pipette. Wherever possible, the formation of air bubbles was avoided. The vessel was then sealed, inverted, and placed in its supporting bottle in the beaker of water which was heated to about 40° C. The dietary liquid, usually serum, took 7–10 min. to reach the temperature of the surrounding water, and after this period of time insects were brought into contact with the membrane.

Air bubbles frequently formed in biological fluids such as blood or serum at this temperature, and it was considered advisable to remove them before the start of feeding, replacing the lost volume with fresh liquid. Bubbles, however, did not usually interfere with successful feeding unless they were large and present in such numbers as to cause mechanical obstruction.

All stages of *Rhodnius* were reared in small culture tubes measuring 2 in. in depth and 1 in. in diameter, with the open ends covered with fine muslin gauze. Bugs were fed by applying the tubes to the warm membrane projecting through the surface of the water in the beaker. Soon after application, the bugs responded to the stimulus of warmth close to them, and immediately commenced probing and feeding.

The temperature of the water-bath during this operation was kept at 38–44° C. by a micro-burner. Lower temperatures (38–40° C.) were favoured by the first three instars and higher temperatures (up to 44° C.) by the later ones. This behaviour is similar to that of *Triatoma* (Nicolle & Mathis, 1941*a*).

III. RESULTS

The behaviour of *Rhodnius* to sources of warmth close to them (Wigglesworth & Gillett 1934) permits the use of artificial feeding for all stages. The data given below refer to normal insects; sterile bugs, that is bugs deprived of their symbiotic micro-organisms, took far less readily to diets other than blood. *Rhodnius* shows little discrimination in choice of food—which indicates that it has a poorly-developed sense of taste. Contrary to some observations (Wigglesworth, 1953, p. 179), all stages of *Rhodnius* fed to repletion on serum without the addition of haemoglobin, and experimental feeding in the laboratory frequently involved the use of horse serum only. (There is, of course, the possibility that some haemoglobin might be present in solution in the serum.)

Bugs were fed on a wide range of liquids, including such unlikely materials as milk. Similarly, wide ranges of diet have been reported for *Triatoma infestans* (Nicolle, 1941; Nicolle & Mathis, 1941*b*), which was induced to feed through a rubber membrane on skimmed milk, washed blood cells, saline solutions and peptone broth.

The readiness with which *Rhodnius* took artificial feeds seemed to decrease with each successive instar. About 80 % of first-instar insects fed to repletion at a single operation; fifth instars required more careful attention and a longer time for feeding to repletion on serum, although they readily took whole blood. Using a single feeding unit, only about 25 % of fifth-instar bugs took a full meal of serum at the first attempt. Once feeding had started, however, bugs usually fed to repletion. There is probably an optimum time for feeding later-stage insects which will give improved results with serum feeding. For example, fifth-instar insects which were fed 3 weeks after the moult took serum more readily than those fed 1–2 weeks or 6–8 weeks afterwards.

Final instar and adult bugs did not feed on *distilled water* or aqueous solutions of glycine or ascorbic acid, although the membranes were repeatedly probed. First and fifth instars and adults fed to repletion on *Ringer's solution*, and all stages on $2\frac{1}{2}$, 5 and 10 % solutions of 'Marmite' in water. Of the large number of first instars fed on this diet, the majority preferred the lowest concentration. Mortality was high 24 hr. after the meal, when symptoms of urine retention, staggering and collapse preceded death. Abdominal distension was marked long after death.

Cow's milk also caused high mortality in first and second instars and adults 24 hr. after ingestion. First instars fed more readily on milk than others. Milk as a diet for *Culex pipiens* has been reported (see Marshall, 1938, p. 247; Myers, 1928). Practically all of 226 mosquitoes fed on skim milk through the layer of cream which acted as the membrane. None were seen to feed on exposed milk.

Horse serum was used regularly for over a year as a base for various diets, such as glucose and haemin with various B vitamins, or with amino acids or antibiotic added. *Rhodnius* frequently took enormous serum meals; one fifth-instar bug ingested 446·1 mg. serum (supplemented with succinylsulphathiazole), and weighed over half a gramme after the meal. The average blood meal taken from the rabbit is 245 mg. Fifth-instar bugs occasionally collapsed after feeding to distension on stored horse serum, but they recovered after several hours. During collapse they lay as they had fallen, with legs tightly drawn up and the probosces often extruded. Frequent discharge of tiny droplets of urine were observed over this period. The insects ultimately recovered without further ill effects. Bugs fed on serum developed normally up to the third stage and then either ceased to moult, or emerged deformed, this being in all probability due to avitaminosis.

Full meals of citrated *rat blood*, oxalated *horse blood* and freshly drawn, whipped ox blood were immediately taken by all stages. The ingestion of stored, oxalated horse blood led to toxic symptoms followed by death. Defibrinated, haemolysed blood, serum or blood cells alone are highly toxic for *Cimex* (de Meillon & Golberg, 1947), horse blood less so than rabbit and human.

To investigate this aspect, normal *Rhodnius* were fed to repletion on stored, oxalated horse blood. None showed immediate symptoms of toxication, but all collapsed within 21 days and died shortly afterwards. The few sterile bugs which fed fairly substantially on the same blood died in 6–18 days. Partial feeding on

horse blood followed by normal feeding on the laboratory host, the rabbit, produced no ill effects, and bugs moulted to the succeeding stage in normal time.

As regards behaviour during feeding, *Rhodnius* showed no reaction if the proboscis came into contact with any moisture which might have formed on the rubber membrane of the feeding unit. On the other hand, any contact of legs with moisture immediately and actively repelled the insect. This was especially the case after contact by the prothoracic legs which would be nearest the source of food or to any moisture present. Bugs were also repelled by moisture from the water-bath, which dampened the blotting-paper strip in the culture tubes.

IV. DISCUSSION

It has been shown that it is possible to feed *Rhodnius* through a rubber membrane on a number of widely differing diets. The number of successful feeds can obviously be increased by the use of more units, which require little equipment and time to erect in the laboratory.

The proposal that the smell of animal skin is partly responsible for the attraction of the bugs to their hosts (Wigglesworth & Gillett, 1934) does not apply here, since bugs were fed for long periods through rubber membranes. These authors found, too, that moisture had no attractive influence, nor was it repellent, for bugs repeatedly probed a warm tube covered with moist voile, and extended their probosces into warm water exposed on a glass slide. This argument applies to contact by proboscis, but the present experiments showed that any contact of legs with moisture repelled the insects. The operative response in all these repulsions seems to be the contact of the legs (probably the tarsi of the legs) with moist surfaces, and not of antennae or proboscis. Such an hypothesis would account for the observations in this paper and those of Wigglesworth & Gillett.

The enormous distension of the *Rhodnius* abdomen with an almost-transparent liquid such as serum is a convenient means of studying heart beats and intestinal peristalsis. The distended gut under magnification shows much detail, and the incorporation of substances with serum before feeding might be of use in studies on the physiology of digestion.

The mortality of bugs fed on 'Marmite' solutions and milk, and the symptoms of urine retention and collapse, parallel the condition of *Glossina morsitans* fed on low concentrations of saline or on water (Lester & Lloyd, 1928) where the insects collapsed after taking full meals of both diets. Occasionally, specimens of *Glossina* survived such meals, but on 'Marmite' and milk there were no recoveries in *Rhodnius*. Lester & Lloyd concluded that water was a tissue poison to tsetse flies, and that death was not caused by prolonged pressure on tracheae and blood vessels, but by the inability of the Malpighian tubes to deal with water, followed by cell destruction due to differences in osmotic pressure. The occasional collapse of bugs after feeding on stored horse serum could be due to some toxic factor produced on storage, or to disorganization of the osmo-regulatory mechanism, but the effects were never fatal as they were after milk and 'Marmite' feeding.

The toxicity of stored horse blood to *Rhodnius* is similar to the condition in

Cimex (de Meillon & Golberg, 1947), but freshly drawn, defibrinated blood has no ill effects when fed to *Rhodnius*. Moreover, bugs fed on this diet moult in about normal time (Clements, 1955). Death by milk and 'Marmite' is rapid and is probably caused by some physical factor; death by stored horse blood is long delayed (about 3 weeks) and must be due to the presence of toxic substances.

V. SUMMARY

1. A simple apparatus for feeding all stages of *Rhodnius prolixus* through a rubber membrane is described.

2. Observations have been made on the behaviour of *Rhodnius* during feeding, and on the effect of different diets on the insect.

3. *Rhodnius* has been shown to feed and grow successfully on freshly drawn rat and horse blood, and on whipped ox blood. Horse serum was readily taken and showed no effect on the development of the bugs until after the third stage, when they either failed to moult or emerged deformed.

Ringer's solution, aqueous solutions of 'Marmite' and cow's milk provided good feeds, though caused mortality after 24 hr. Stored oxalated blood killed bugs 21 days after feeding. No feeding took place when diets of water, glycine and ascorbic acid were offered.

This research formed part of a study for the degree of Ph.D. of the University of London and was carried out under the late Professor P. A. Buxton, F.R.S., whose valuable comments were much appreciated. I wish to thank Mr S. A. Smith for technical assistance and suggestions given during the work. It is a pleasure to acknowledge the generous financial support of the South African Council for Scientific and Industrial Research.

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With 1 *text-figures*
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Studies on *Rhodnius prolixus*: growth and development of normal and sterile bugs, and the symbiotic relationship

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I. INTRODUCTION

The blood-sucking bug, *Rhodnius prolixus*, has as its micro-symbiont a typical Actinomycete, *Nocardia rhodnii* (the *Actinomyces rhodnii* of Erikson, 1935), which exists extracellularly in the gut and is essential to the well-being and complete development of its host. The symbionts are acquired soon after hatching, by contamination from the faeces of the parent bugs (Brecher & Wigglesworth, 1944). Infected bugs are easily deprived of their micro-organisms by surface-sterilization of the eggs, and in this way sterile cultures of the insects can be obtained for comparative studies with normal, infected bugs. At an early stage of an investigation into certain biochemical aspects of sterile bugs, it became apparent that further information was required on the growth and development of this group for comparison with that of normal insects. The growth and development of both groups were therefore compared.

A certain amount of confusion has arisen regarding the symbiotic state in *Rhodnius* and the related blood-sucking bug, *Triatoma infestans*, due mainly to problems concerning the identification of the micro-organisms present. The matter is discussed at the end of this paper.

The sensitivity of *Nocardia rhodnii* to antibiotic substances was determined in order to fill a small gap in the existing knowledge of the symbiont.

II. MATERIALS AND METHODS

(*a*) *Rearing of sterile cultures*

The methods of rearing and feeding *Rhodnius prolixus* in the laboratory have been described by Buxton (1930). Sterile cultures were obtained after surface-sterilization of eggs with 5% gentian violet, and by feeding the hatched larvae on a rabbit's ear wiped over with gentian violet (Brecher & Wigglesworth, 1944).

As far as it was possible, every precaution was observed to maintain the sterility of the cultures, although the insects were not fed on separate rabbits, and this could be a potential source of infection. Periodic examinations were made to confirm the sterility of the culture. These included microscopical investigation and, more usually, bacteriological culture on blood agar. Sterile cultures were kept at 27° C. in a room separate from normal, infected stock.

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(*b*) *Culture of micro-organisms*

Nocardia rhodnii is conveniently cultured on plates of serum agar (Erikson, 1935), and on blood agar, when at 30° C. whitish to buff colonies appear after 48 hr. (Brecher & Wigglesworth, 1944). Ethanol (70%) and tincture of iodine were preferred to the lysol used by the above authors as antiseptic substances for the cleansing of the abdominal surfaces of insects before withdrawal of ingested blood. The sterilized abdominal surface was punctured with a Pasteur pipette and a sample of ingested blood withdrawn for cultivation on blood agar plates.

III. RESULTS

(*a*) *Comparison of normal and sterile* Rhodnius

Growth and development

Cultures of normal and sterile insects were weighed before and after separate feeding on the rabbit. Growth curves are shown in Fig. 1.

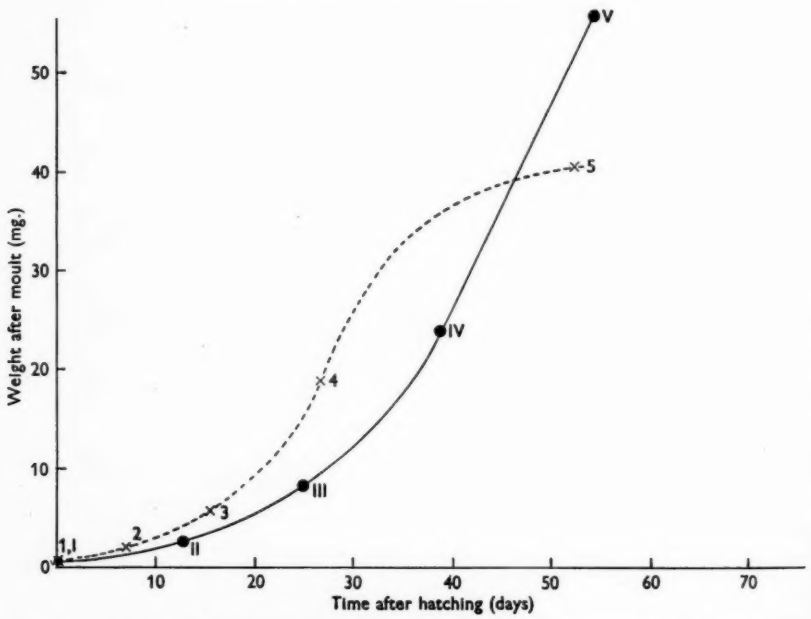

Fig. 1. Growth curves of normal and sterile *Rhodnius prolixus*. ----, sterile; ——, normal.

Brecher & Wigglesworth (1944) obtained a few adults from a culture deprived of *Nocardia*, but these proved incapable of reproduction. In the present study, out of several hundreds of sterile insects reared, no adults were obtained and only six final instar bugs, three of which died in the moult to the adult stage, had the characteristic deformity observed by Geigy, Halff & Kocher (1953) in *Triatoma infestans*. Baines (1956) also found that no sterile nymphs completed metamorphosis.

Brecher & Wigglesworth found that sterile insects grew and moulted normally until the fourth or fifth instar, after which moulting was delayed, or failed, in spite of repeated blood meals. These findings were confirmed by Baines (1956), although in the present study significant differences in weight and duration of instars between sterile and normal bugs were observed. In the later instars, not only did sterile bugs fail to moult, but the mortality of the culture during moulting caused severe depletion of stock (Table 1).

Table 1. *Moulting, and mortality during moulting, of fourth-instar* Rhodnius (*sterile for* Nocardia rhodnii)

	No. of insects used	Percentage moulted	Percentage mortality during moulting
1st feed	78	43·4	37·1
2nd feed	50	40·0	40·0

Table 2. *Body weights of normal and sterile* Rhodnius*

Instar	Normal	Sterile
First	0·43	0·42
Second	2·56	1·81
Third	8·06	5·07
Fourth	23·70	18·78
Fifth	55·84	40·83

* In milligrammes, taken before blood meal. Twenty bugs used for each determination except for first instars, when thirty normal and forty-five sterile were used.

In normal bugs, mortality before or during moulting is rare, and diseased specimens were never found in cultures (see also Buxton, 1930). After repeated blood meals and subsequent failure to moult, the condition of sterile insects progressively deteriorated, the response to the host diminished and many bugs refused to feed. A single fifth-instar bug which survived for six months took four blood meals, each successively smaller than the last. It consistently refused another meal, and died soon after.

Statistical analysis of the weights of normal and sterilized insects immediately after ecdysis (Table 2) shows that the last three nymphal instars of the sterilized group weigh significantly less than the normal ones. There is little use in trying to distinguish between the two groups at the first instar, nor, with such small samples, the ones in the second instar. Normal first-instar bugs took an average blood meal of 4·40 mg. compared with 3·75 mg. taken by sterile bugs (mean of 20 weights taken); this was the first weight difference to be observed between the two cultures.

Eggs which were surface-sterilized with gentian violet hatched at the same time as untreated eggs, but the duration of each instar differed appreciably (Table 3).

Sterile insects completed the first three stages of their life histories in less time than did normal ones. At the 4th stage, however, the detrimental effect of nutritional deficiencies came into play, and the sterile culture either failed to moult, or

moulted over erratic periods of time ranging from 19 to 46 days, a difference highly significant when compared with the normal group. These results contrast with previous observations that sterile *Rhodnius* grow and moult normally until the fourth of fifth instar (Brecher & Wigglesworth, 1944; Wigglesworth, 1952), or until the third instar (Baines, 1956).

Table 3. *Duration of instars of normal and sterile* Rhodnius *in days, at* 26–27° C.

Instar	Normal	S.E.	Sterile	S.E.
First	12·70	0·596	6·98	0·067
Second	12·18	0·229	8·53	0·667
Third	13·87	0·369	11·14	0·865
Fourth	15·54	1·06	19–46	—
Fifth	25·82	1·78	—	—

(*b*) *Characteristics of the symbiotic micro-organisms in* Rhodnius

Nocardia rhodnii was cultured on 5% blood agar at 30° C., and white to buff colonies became visible in 24–48 hr. Microscopical examination of the coccoid forms after staining with Giemsa and Gram's stains confirmed the identity of the culture. The organism was cultured on simple, synthetic media (phosphate and ammonium salts, trace metals, glucose and lactate), with no growth factors present. It grew equally well on ordinary nutrient agar. Such cultural characteristics are typical of Actinomycetes (Waksman, 1953).

Sensitivity of Nocardia rhodnii *to antibiotics*

Pure cultures of *N. rhodnii* maintained on glucose broth slants were transferred to blood agar and subsequently cultivated on nutrient broth for 24–48 hr. at 30° C. An aliquot of the liquid culture was then spread uniformly over the surface of a blood-agar plate and the efficacy of five antibiotics tested by the application of the horizontal diffusion method, using the 'Sentest' unit of Evans Medical Supplies. The results recorded in Table 4 show that terramycin is the most effective inhibitor of *N. rhodnii* in pure culture. Aureomycin and chloramphenicol are, however, almost as effective. This is in contrast to the condition in *Triatoma* where of the three antibiotics, chloromycetin, aureomycin and terramycin fed in serum to infected bugs, terramycin only was effective in inhibiting the development of the symbionts (Geigy, Halff & Kocher, 1954).

A number of normal fifth-instar *Rhodnius* were allowed to take small quantities of serum supplemented with succinylsulphathiazole in such proportions that a wide range of the drug would be ingested by different bugs. They were then allowed to feed to repletion on the rabbit. Amounts of 60 μg. antibiotic and over had lethal effects. Doses from 7 to 16 μg. were tolerated, and treated bugs either failed to moult or died in ecdysis long after untreated bugs had moulted and in a manner analogous to the condition in sterile bugs. Microscopical examination of ingested blood of two specimens showed no evidence of *Nocardia rhodnii*. The remains of the blood meal taken from one bug 47 days after feeding was inoculated on the

5% blood agar and kept at 30° C. for 48 hr., when a pure culture of *N. rhodnii* was obtained, but one much reduced in density and growth when compared with a control colony from untreated bugs. When *Triatoma infestans* was fed artificially on a diet of guinea-pig serum with terramycin (10 mg./ml.) added (Geigy *et al.* 1954), no symbionts were found in the gut up to 19 days after the meal. Observations similar to the above were made on bacteroids in penicillin-treated cockroaches (Brues & Dunn, 1945), who found that low concentrations of penicillin greatly reduced the number of micro-organisms, but after the insects were allowed to live normally for a time, the bacteroids showed a gradual increase in number.

Table 4. *Inhibition of* N. rhodnii *by antibiotics*

Antibiotic	Concentration*	Diameter of zone of inhibition (mm.)
Penicillin	0·5	—
	2·5	7
Streptomycin	20	—
	80	10
Chloramphenicol	40	16
	100	20
Aureomycin	10	15
	100	20
Terramycin	10	16
	100	22

* All in μg. except penicillin (in i.u.).

IV. DISCUSSION

As far as the growth and development of sterile *Rhodnius* are concerned, the experimental results in some respects conform with those of Brecher & Wigglesworth (1944) and Baines (1956), but differ in others. No adults were obtained during the investigation—in fact, fourth-instar mortality is so high that sterility for *Nocardia* should be guarded against in the rearing of stock cultures. Before the first blood meal, normal and sterile insects weigh about the same (0·43 and 0·41 mg. respectively). The first feed makes the first difference, normal bugs taking 1·2 times more blood than sterile ones. The pattern of growth and development of sterile insects seems to be laid down at the first blood meal, after which sterile insects become progressively smaller and lighter than normal ones.

Comparative studies of the duration of the life histories of both groups show that at the same temperature sterile insects take less time to reach the fourth instar than normal ones. This suggests that some sort of competition is taking place between micro-organisms and the parent bug. This is unlikely to be for vitamins since Actinomycetes do not require preformed growth factors in culture media (see Waksman, 1953, and the present study). In addition, normal *Rhodnius* grow and develop quite normally up to the third instar on serum alone which has low levels of vitamins.

Two explanations have been suggested to explain why sterile insects are affected at the fourth and fifth instars and not earlier (Wigglesworth, 1952). They are that the ingestion of greater amounts of blood in proportion to body weight ingested by the earlier instars, provides them with sufficient of the missing factor, or that this factor is carried over in the egg and so suffices the insect for several months. The successful development up to the third stage of normal bugs on serum alone, which is extremely low in vitamin content, seems to support the second explanation. Further suggestive evidence comes from the detection of vitamins in insect eggs, e.g. folic acid in eggs of *Bombyx mori* (Busnel, Levy & Polonovski, 1950) and of riboflavin in *Locusta* and *Shistocerca* (Goodwin & Srisukh, 1951).

There exists at the present time some confusion regarding the microflora of *Rhodnius* and the related blood-sucking bug *Triatoma infestans*. *Nocardia rhodnii* has been consistently found in pure culture in *Rhodnius* (Wigglesworth, 1952; Goodchild, 1955; Baines, 1956; and confirmed in the present study), although Bewig & Schwartz (1954, 1955, 1956) report the presence of an accompanying coccoid-like organism which they identify as *Streptococcus liquefaciens*. They ascribe its previous non-detection as being due to confusion of the organism with a stage of the *Nocardia rhodnii* also present. This accompanying organism was found in 50 % of the bugs examined. Apart from these two organisms, no others were found by these authors.

On the other hand, *Triatoma infestans*, as Goodchild's study (1955) has shown, can have micro-organisms other than *Nocardia rhodnii*. In fact, it generally did, and when *N. rhodnii* was present in the gut it was found to occur in pure culture because of its antibiotic activity (see also Wigglesworth, 1952).

Four fairly common organisms were isolated from *Triatoma* by Goodchild (1955), *Bacterium*, *Corynebacterium*, *Staphylococcus* and *Streptomyces* spp. The commonest species isolated was the *Corynebacterium* (diptheroid) sp. near *hofmanii* which was found within cell vacuoles in the intestine. *Streptococcus faecalis* has also been isolated from this bug (Brecher & Wigglesworth, 1944), and Weurman (1946) reports the isolation of other organisms. There is, therefore, no one organism consistently present in *Triatoma*.

The identity of the organism described by Geigy *et al.* (1954) as being symbiotic in *Triatoma*, on the basis of its hyperproduction of folic acid, needs clarification. These authors describe the organism of Brecher & Wigglesworth (1944) (which is *Nocardia rhodnii*), as '...des symbiotes analogues, quoique d'un type un peu différent' to what they isolated and described as the symbiont in *Triatoma*. Doubt has been cast by Bewig & Schwartz as to whether the organism of Geigy *et al.* is indeed a symbiont. Bewig & Schwartz report having identified this latter organism as *Streptococcus liquefaciens*, an organism accompanying *Nocardia rhodnii*, and which they showed to be non-symbiotic. They suggest that the figure for folic acid production reported by Geigy *et al.* (1954) is too high. Furthermore, cross-infection of sterile *Triatoma* can be accomplished with the *Nocardia rhodnii* of *Rhodnius* (Brecher & Wigglesworth, 1944) but not with *Streptococcus liquefaciens*, neither from *Rhodnius* to *Triatoma* nor vice versa.

That the organism of Geigy *et al.* is distinctly different to *Nocardia rhodnii* can

be inferred from difference in cultural characteristics (c.f. Eriksen, 1935), the vitamin requirements of the organism (c.f. Waksman, 1953) and by its different responses to antibiotic substances as demonstrated in the present work.

One must emphasize the care that is necessary to make the correct identification of the micro-organisms present. Only then can the contribution made by these forms be determined by bacteriological, chemical or biological methods such as nutritional experiments. The micro-organisms in *Triatoma* have been shown to be of such variety, and the consistent presence of the same forms so uncertain, that comparisons with the symbiotic condition in *Rhodnius* cannot be made unless both have *Nocardia rhodnii* to the exclusion of all others.

V. SUMMARY

1. The growth and development of normal and sterile (i.e. symbiont-free) *Rhodnius prolixus* are compared. The last three instars of sterile bugs weigh less than normal ones. Sterile insects completed the first three stages of their life-histories in less time than normal insects, and at the fourth stage, nutritional deficiencies had profound effects on their further development.

2. The sensitivity of the *Rhodnius* symbionts, *Nocardia rhodnii*, to five antibiotic substances is determined. Terramycin, aureomycin and chloramphenicol are effective inhibitors.

3. The symbiotic condition in *Rhodnius* and the related bug, *Triatoma infestans*, is discussed.

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